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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by the law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1980.

Phase I–II Study of Piperazinedione in Adults With Solid Tumors and Acute Leukemia [1,2]

Robert S. Benjamin,[3,4,*] Michael J. Keating,[3] Manuel Valdivieso,[3] Kenneth B. McCredie,[3] Robert A. Livingston,[3,5] Michael A. Burgess,[3] Victorio Rodriguez,[3,6] Gerald P. Bodey,[3] and Jeffrey A. Gottlieb[3,†]

SUMMARY

Piperazinedione was administered to 79 patients with solid tumors on an intermittent schedule with single doses of 1.5–36 mg/m^2. Courses were usually repeated at 4-week intervals. Twenty-five patients with leukemia were treated at doses of 18–36 mg/m^2 (occasionally for 2 successive days) every 1–4 weeks. Of 48 evaluable patients with malignant melanoma, three (6%) achieved partial remission and nine (20%) had stable disease. Eight of 17 (47%) patients with adenocarcinomas and one of two (50%) patients with lymphomas also had stable disease. Six of 14 (43%) patients with acute myelogenous leukemia showed hematologic improvement, as did one of 11 (9%) patients with blast cell crisis of chronic myelogenous leukemia. The principal toxic effect was myelosuppression, which occurred in 69% of the patients with solid tumors. Profound bone marrow aplasia occurred in 19% of the patients, resulting in six deaths (8%). Risk factors for marrow aplasia included extensive prior therapy, prior nitrosoureas, cumulative toxicity from piperazinedione, and abnormal liver function tests. The recommended doses for further studies are 9 mg/m^2 for patients with risk factors for marrow aplasia, 12 mg/m^2 for patients with prior therapy, 15 mg/m^2 for previously untreated patients, and 24–36 mg/m^2 for patients with acute leukemia.

[Cancer Treat Rep 63:939–943, 1979]

Piperazine derivatives were introduced into clinical trials as antitumor agents in the 1950s. The most widely studied derivatives included pipobroman and piposulfan, which reached clinical trials because they were effective anthelmintic agents and showed efficacy in the treatment of chronic leukemia and polycythemia vera (appearing to act as alkylating agents) (1–4). The next piperazine derivatives to receive clinical investigation were ICRF-154 and ICRF-159. Although ICRF-154 was not shown to have either toxic or therapeutic effects in patients with acute leukemia, ICRF-159 revealed evidence of activity in patients with various hematologic malignancies (5).

The efficacy of these piperazine derivatives has led to interest in similar compounds. A crystalline antibiotic derived from the fermentation products of a strain of *Streptomyces* has received intensive preclinical and toxicologic evaluation. This drug, referred to as piperazinedione (fig 1), has shown activity against L1210 and P388 leukemias, B16 melanoma, Walker 256 carcinosarcoma, Lewis lung carcinoma, and ependymoblastoma. Although the mode of action of this drug has not been clearly defined, it has demonstrated an ability to inhibit DNA synthesis and may act, at least in part, like an alkylating agent (6). In some studies with ip inoculated L1210 leu-

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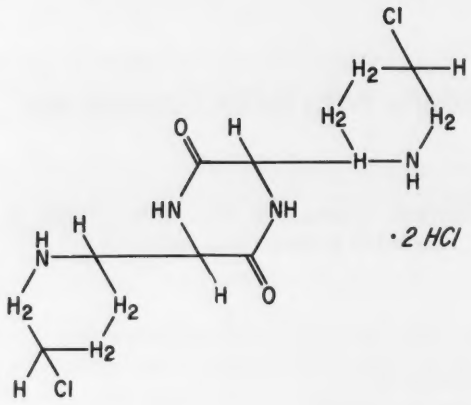

FIGURE 1.—Structure of piperazinedione.

kemia resistant to cyclophosphamide, there was moderate cross-resistance to piperazinedione (7,8), although other strains resistant to classic alkylating agents and nitrosoureas are sensitive to piperazinedione (9).

Preclinical investigations revealed little schedule dependency with piperazinedione; thus, we investigated the drug in single large injections repeated after bone marrow recovery. Toxic effects of this drug in animals included pancytopenia, lymphoid depletion, hemorrhage, necrosis, atrophy of the intestinal mucosa, and atrophy of the gonads.

Based on the toxic dose low (TDL) of 0.9 mg/m^2/day × 5 in both dogs and monkeys, it had been recommended that the starting dose in man for a 5-day schedule would be safe at one third of this level, or 0.3 mg/m^2/day × 5. Although the TDL for a single injection was not available, from studies of the highest nontoxic dose (3.8 mg/m^2) and the toxic dose high (8 mg/m^2), it could be anticipated that the TDL for dogs receiving single doses would be in the vicinity of 4.5 mg/m^2. Based on the animal data, we initiated our phase I study at a dose of 1.5 mg/m^2 administered as a single dose.

MATERIALS AND METHODS

One hundred and four patients (79 with solid tumors and 25 with leukemia) were entered in the study. Initial dosage escalations were performed by 100% increments until toxicity was observed. Further escalation was performed according to the method of Gottlieb (10), starting at a dose of 1.5 mg/m^2 and escalating to a dose of 36 mg/m^2. Patients with solid tumors were treated primarily with doses of 12–15 mg/m^2 every 4 weeks. Patients with leukemia received doses of 18–36 mg/m^2, usually given as a single dose (repeated in some, however, on 2 successive days) every 1–4 weeks. For patients with solid tumors, a complete remission (CR) was defined as the disappearance of all clinical evidence of active tumor and symptoms for a minimum of 4 weeks. Partial remission (PR) was defined as a ≥ 50% decrease in the sum of the products of the perpendicular diameters of all measured lesions. No simultaneous increase in the size of any lesion or the appearance of any new lesions could occur, and the remission had to be maintained for at least 4 weeks. Stable disease (SD) was defined as tumor regression not qualifying for PR but lasting at least 4 weeks or a steady state of response not qualifying for increasing disease but lasting at least 8 weeks. Increasing disease was defined as an increase of at least 25% in the size of any measured lesion or the appearance of any new lesions.

For patients with acute leukemia, CR was defined as the return of bone marrow and peripheral blood cell counts to normal for a minimum of 4 weeks. The bone marrow had to contain < 5% blast cells. For patients with acute leukemia, PR was defined as the return of the peripheral blood cell counts to normal with 5%–30% blast cells in the bone marrow. Hematologic improvement was defined as any lesser degree of objective improvement in hematologic status.

RESULTS

Efficacy

Hints of antitumor activity in terms of stabilization of disease were noted by the third dose escalation. Since only three patients received only doses below this level, all patients were considered eligible for phase II evaluation of response.

Malignant Melanoma

The 52 patients with malignant melanoma constituted our largest subgroup. Four patients died within 2 weeks of therapy and have not been evaluated for response. Forty-six of the 48 evaluable patients had received prior therapy with DTIC, a nitrosourea, immunotherapy, and/or other drugs. Three patients (6%) with soft tissue disease achieved a PR of 1–2 months' duration. One of these patients had received no prior therapy. Nine additional patients (20%) had SD of 2–4 months' duration (table 1).

Other Solid Tumors

Twenty-seven patients with other solid tumors received piperazinedione (table 1). Although no patient achieved PR, ten (37%) had SD of 1–4 months' duration (median, 2). The best results were achieved in patients with adenocarcinomas.

Acute Myelogenous Leukemia (AML)

Fourteen previously treated patients with AML received piperazinedione. No patient achieved CR or PR,

TABLE 1.—Piperazinedione in patients with solid tumors and leukemia

Diagnosis	No. of—			
	Patients treated	PRs	Improvements or SDs	Treatment failures
Melanoma	52	3	9	36
Adenocarcinoma	17	0	8	9
Gastrointestinal	8	0	4	4
Unknown primary	3	0	1	2
Thyroid	2	0	0	2
Breast	1	0	1	0
Prostate	1	0	1	0
Renal	1	0	1	0
Lung	1	0	0	1
Squamous cell carcinoma	5	0	0	5
Head and neck	4	0	0	4
Lung	1	0	0	1
Sarcoma	3	0	0	0
Lymphoma	2	0	1	1
Hodgkin's disease	1	0	1	0
Nodular poorly differentiated lymphocytic lymphoma	1	0	0	0
AML	14	0	6	8
CML in blast cell crisis	11	0	1	10

but six (43%), including one in leukemic crisis, had hematologic improvement with clearance of peripheral blast cells and some effect on the bone marrow. In two (14%) of these patients, bone marrow differential improved and in the other four patients the marrow was rendered aplastic. In six other patients, the peripheral blood blast cells disappeared for 27–91 days (median, 37), but there was no effect on the bone marrow.

Blast Cell Crisis of Chronic Myelogenous Leukemia (CML)

Eleven patients with CML received piperazinedione (18–36 mg/m^2 every 1–4 weeks) for blast cell crisis. Ten patients were positive for the Philadelphia chromosome. Peripheral blood cell counts returned to normal and the bone marrow blast cell plus promyelocyte percentage decreased to < 50 in one patient. In nine other patients, blast cells disappeared from peripheral blood smears. However, there was only a transient effect on the bone marrow in most patients, although persistent marrow aplasia was attained in one.

Toxicity

Hematologic toxicity was evaluated only in the 75 patients with solid tumors who survived > 2 weeks. Leukopenia or thrombocytopenia (or both) occurred in 52 (69%) patients. Blood cell counts were available at least weekly on 47 patients, and details of their hematologic toxicity are shown in table 2. Maximum myelosuppression was reached 17 days after drug administration, with recovery usually complete before 4 weeks. In some patients recovery was delayed for 6–8 weeks. Fourteen (19%) patients had profound bone marrow aplasia, and six (8%) of these died as a result of therapy. Severe bone marrow toxicity was not clearly dose-related at doses of $\geq$ 12 mg/m^2; it was more frequent in patients with extensive prior therapy, any prior exposure to nitrosoureas, cumulative exposure to piperazinedione at 3–4-week intervals, or abnormal liver function tests. One or more of these risk factors were present in 11 of 14 patients with severe myelotoxicity. Nonetheless, bone marrow toxicity was occasionally unpredictable and unrelated to these risk factors in three patients. Based on the timing and severity of myelosuppression (table 2), for future phase II trials we currently recommend therapy at 28-day intervals at doses of 9 mg/m^2 for patients with the risk factors noted above, 12 mg/m^2 for other patients with prior therapy, and 15 mg/m^2 for good-risk patients without prior therapy.

Other toxic effects were evaluated in all 98 patients. These included nausea and vomiting in 26 (27%) patients, drug fever in six (6%), and increased prothrombin time in four (4%). Other toxic effects commonly associated with antineoplastic agents were not observed.

The one patient with a solid tumor who received a dose of 36 mg/m^2 of piperazinedione had persistent nausea, vomiting, and diarrhea for 4 days. By Day 6, her wbc count had decreased to 700/μl, her platelet count had decreased to 10,000/μl, and her hemoglobin count had decreased to 10.6 g/dl. She died on Day 83 after seven

TABLE 2.—Piperazinedione myelosuppression

Dose (mg/m²)	No. of patients evaluated	No. of patients with— Wbc counts of < 4.0 × 10³/μl	Granulocyte counts of < 2.0 × 10³/μl	Platelet counts of < 100 × 10³/μl
1.5	3	0	0	0
3	2	0	0	0
6	3	0	0	0
12	19	9 (1.9;0.8–3.4)*	9 (1.1;0–2.0)	10 (64;9–99)
15	8	6 (2.2;1.6–3.1)	6 (1.5;0–7.2)	4 (47;15–80)
18	7	6 (1.7;1.0–2.6)	6 (1.0;0.7–1.9)	5 (36;5–90)
24	5	4 (1.1;1.0–3.6)	4 (0.3;0.1–2.0)	4 (33;25–99)
36	1	1 (0.6)	1 (0)	1 (10)

*Nos. in parentheses = median counts of patients with cytopenia; range.

documented episodes of septicemia. Her wbc count was 600/μl, her platelet count was 12,000/μl, and her hemoglobin count was 6.9 g/dl. An autopsy showed hypoplastic bone marrow, lymphoid depletion, necrotizing mucositis, and disseminated toxoplasmosis. There was no other clinical or pathologic evidence of additional organ impairment.

DISCUSSION

Piperazinedione is capable of inducing objective regressions in human cancers as well as in a large variety of animal tumor models. Although the therapeutic results in our phase I–II trial are far from striking, the advanced stage of disease and degree of prior therapy of our patients must be taken into account in interpreting the data. Phase II studies based on a preliminary analysis of this study have been published for Hodgkin's disease (11), lymphomas (11), myeloma (11), breast cancer (12), and renal carcinoma (13). Five of seven patients with Hodgkin's disease responded as did three of six with nodular poorly differentiated lymphocytic lymphoma. No responses were seen in the patients with other lymphomas, myeloma, or renal carcinoma, and only three of 58 patients with breast carcinoma responded.

The major toxic effect of piperazinedione is severe myelosuppression which can be irreversible. Myelosuppression was also noted in subsequent phase II evaluations (11–13) and in the other phase I study by Pratt et al (14), where piperazinedione was administered on a 5-day schedule with recommended phase II doses of 3 mg/m² every day × 5 for those patients with solid tumors and 5 mg/m² every day × 5 for those patients with acute leukemia. Myelosuppression may limit its ultimate use in combination chemotherapy. Presant and Klahr (15) reported severe myelosuppression, especially thrombocytopenia, in three of six patients treated in a phase I combination study at doses of 5 mg/m² (piperazinedione), 500 mg/m² (cyclophosphamide), and 500 mg/m² (DTIC); minimal myelosuppression was seen, however, in patients treated with doses 20% lower. Two of the three patients had received prior radiation. One wonders if the other risk factors noted in our study—prior nitrosoureas, extensive prior therapy, or abnormal liver function—were present in the third patient. The absence of other major side effects from piperazinedione, even at very high doses, suggests that it may prove to be a useful drug in preparing patients for bone marrow transplantation, and we are currently exploring its use in this situation. Similarly, it might be an effective agent for the treatment of CML in the chronic phase or for maintenance therapy for acute leukemia.

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Thymidine and Thymine in Biologic Fluids During High-Dose Infusions of Thymidine in Mice, Monkeys, and Man[1]

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SUMMARY

Measurements were made to determine if millimolar thymidine (TdR) concentrations, shown to have antitumor activity in vitro, could be attained in vivo. An adaptation of an ion-exclusion liquid chromatography method was used to quantitate TdR and thymine in various biologic fluids. Millimolar concentrations of TdR were attained in plasma of mice, monkeys, and man by constant infusions of large quantities of TdR (~ 80 g/m^2/day). Cerebrospinal fluid concentrations of TdR at equilibrium were one tenth of the plasma concentration in the monkey. The primary route of removal of TdR at these high plasma concentrations is kidney clearance of intact TdR. Metabolism, which plays a major role in the clearance of TdR at micromolar concentrations in plasma, appears to be saturated at millimolar concentrations of TdR.

[Cancer Treat Rep 63:945–949, 1979]

High concentrations of thymidine (TdR), approximately millimolar, have been shown to affect a variety of cell types in vitro (1–3). Several reports have suggested that TdR may have a selective antitumor action both in vitro at high concentrations (millimolar) and in vivo at very high frequent doses (4–6). Available experimental data on pharmacokinetic behavior of TdR indicates that it is rapidly cleared from the mammalian host (7–9). Compounds that exhibit this type of in vivo kinetic behavior, even if capable of killing cultured cells in vitro upon prolonged exposure, often have no effect on tumor growth in vivo (10). One reason is that they are often administered by multiple injections and therapeutic concentrations are not sustained for sufficient periods of time. The purpose of these investigations was to determine whether TdR concentrations that have been shown to affect cells adversely in vitro can be attained and sustained for prolonged periods in vivo.

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MATERIALS AND METHODS

TdR was obtained from the Sigma Chemical Co (St. Louis, Mo) for animal experiments and from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md) for human studies. Nude mice bred and maintained in pathogen-free conditions were used (11). The rhesus monkey was obtained from the National Institutes of Health animal farm. The human subjects were a normal volunteer (84 kg) and a terminal cancer patient (80 kg) at the St. Joseph Hospital, Houston, Tex.

The assay system for TdR and thymine (T) is an adaptation of the method used for the measurement of nucleosides in hydrolysates of RNA and DNA (12). Plasma is precipitated under ice-cold conditions with an equal volume of 1 N perchloric acid. The supernatant is adjusted to pH 9–11 by addition of 4 N KOH. It takes 2 days at 5°C for all the potassium salt to completely precipitate and for the pH to stabilize. This supernatant is filtered and ≤ 20 µl is injected into a high-pressure liquid chromatograph (HPLC). Urine and cerebrospinal fluid (CSF) samples are first diluted at least ten times and then filtered (0.4-µm type HA with type AP prefilter, Millipore) at room temperature prior to HPLC analysis. The HPLC column is 50 cm long × 4 mm (internal diameter), packed with Aminex A-7 cation exchange resin, particle size 7–

TABLE 1.—Plasma TdR and T concentrations during constant sc infusion of TdR in normal nude mice at 0.5 g/24 hrs/mouse (~ 0.6 g/kg/hr)*

Time after start of infusion (hrs)	No. of mice	TdR (mM ± SD)	T (mM ± SD)
24	8	0.78 ± 0.24	0.29 ± 0.07
66	4	0.43 ± 0.28	0.32 ± 0.04
96	10	0.27 ± 0.08	0.20 ± 0.02

*Mean body weight = 34 ± 4 (SD) g.

11 microns (Bio-Rad Laboratory, Richmond, Calif). The eluant is $(NH_4)_2CO_3$ adjusted to pH 9.78 with NH_4OH. The pH must be adjusted daily by addition of NH_4OH. Ambient conditions of 25°C at a pressure of 1000 psi result in a flow rate of 0.215 ml/minute. Peak heights of TdR and T at elution times of 23 and 37 minutes respectively were measured by absorbance at 254 nm in an 8-μl flow cell. Most of the other ultraviolet-absorbing substances present in plasma and urine extracts eluted prior to T. Peak heights are proportional to the quantity of TdR and T in the solution injection, if ≤ 20 μl is used. Standard curves were linear in the utilized range. With an injection volume of 20 μl, solutions with TdR concentrations below micromolar are undetectable.

RESULTS

Nude mice.—A system for continuous sc infusion of TdR in nude mice has been developed (13). Administration of several courses of TdR in Eagle's minimal essential medium at 0.4–0.9 g/kg/hour (15–20 ml/kg/hour) for from 72 to 140 hours results in partial to total regression of human tumors growing in these mice (B. Giovanella, personal communication). Table 1 reports the plasma TdR and T concentrations in normal nude mice given similar infusions of TdR in separate experiments. It is seen that TdR concentrations decrease with time whereas T concentrations remain fairly constant with time. This phenomenon cannot be adequately explained at this time but it has been observed that deterioration of the sc infusion region, with significant leakage, occurs following sc infusions for ≥ 5 days. Subsequent studies in man with iv infusions show no such decrease with time of infusion.

Man.—Initial trials were made with oral ingestions of TdR (40 and 80 g) in a syrup suspension. Plasma TdR did not rise above 30 μM whereas T in plasma approached 0.5 mM. It was also noticed that removal of TdR was more rapid compared to T. Eight hours after ingestion, TdR was undetectable in plasma whereas T remained above 0.1 mM. In one trial in which 80 g of TdR was suspended in milk, a severe diarrhea in the subject occurred following oral ingestion. Oral administration of TdR did not appear to be a promising way to achieve high TdR concentrations in plasma and was not pursued further. The next empirical trials were with solutions of TdR (≤ 30 mg/ml) made isotonic with saline, filtered to sterilize, and then infused iv. Results are illustrated in figure 1a. It can be seen that plasma TdR concentrations approach those achieved in the nude mouse. It is also apparent from these data that the removal rate of TdR after cessation of infusion is much more rapid than that of T. Figure 1b illustrates the results obtained with an 8-hour infusion. These data show that plasma concentrations of millimolar TdR are exceeded. The half-lives for TdR and T of 80 and 235 minutes respectively after 8 hours of infusion are much longer than the half-lives of 10 and 35 minutes respectively after 30 minutes of infusion. Figure 1c illustrates the results with 24 hours of infusion. A plateau TdR concentration is reached at about 12 hours. The half-life for TdR, with the limited data available, was approximately 100 minutes and the half-life for T was much longer but could not be determined from the limited data. Figure 2 illustrates results with 120 hours of infusion in a patient and indicates that plasma concentrations of TdR and T close to millimolar are attained. Half-lives based on a minimal amount of data were 100 minutes for TdR and 240 minutes for T. These values are consistent with the long half-lives seen after 8 hours of infusion in a normal subject.

Monkey.—Since many tumors metastasize to the brain, it seemed important to determine what CSF concentrations of TdR were attainable when millimolar plasma concentrations were achieved. An infusion rate calculated to give millimolar TdR in plasma was estimated from the earlier human studies. Use of the concept that clearance in different mammalian species can be related to surface area, or body weight to an exponential power (14,15), gave us an infusion rate of 240 mg/kg/hour for a 4.8-kg monkey. The infusion solution (TdR in 0.6% saline) was administered via a catheter in a saphenous vein. Figure 3 illustrates some of the data. The plasma concentrations of TdR and T are close to millimolar. The concentrations of TdR in the CSF equilibrate at a level tenfold lower than in plasma, whereas the concentration of T in the CSF slowly approaches the concentration of T in the plasma. Quantitation of TdR and T in total urine collections enabled kidney clearances to be calculated:

$$\text{Kidney clearance (ml/minute)} = \frac{\text{urine (mmols/ml)} \times \text{vol (ml/minute)}}{\text{plasma (mmols/ml)}}.$$

Total-body clearance at steady-state plasma concentration when input equals output was calculated as:

$$\text{Total body clearance (ml/minute)} = \frac{\text{input (mmols:minute)}}{\text{plasma (mmols/ml)}}.$$

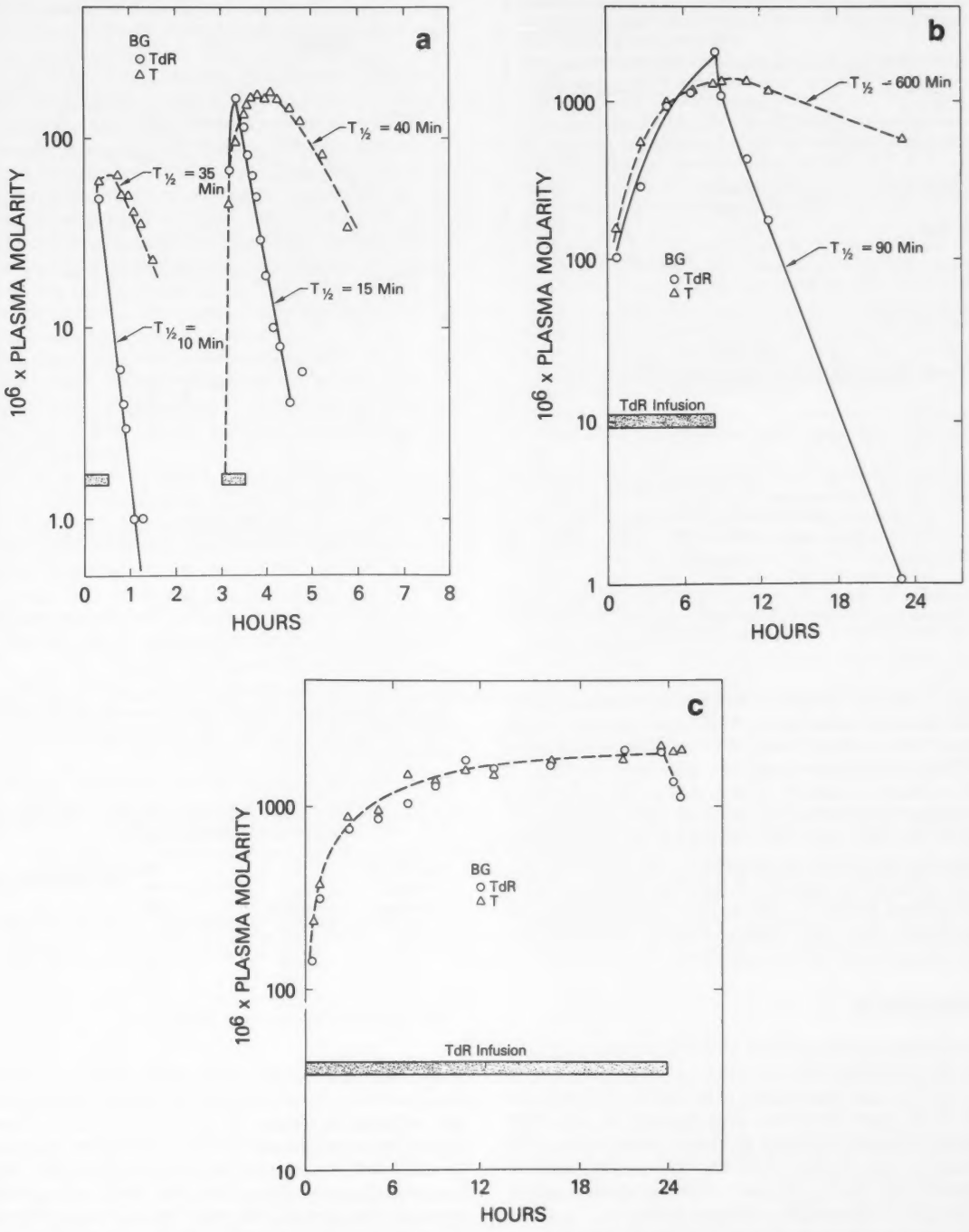

FIGURE 1.—a. Infusion of TdR iv into a normal human subject (84 kg). Initial rate = 48 mg/kg/hr for 30 mins. Total volume = 500 ml of isotonic saline. Second infusion at 3 hrs was identical to the first except that the TdR infusion rate was doubled to 95 mg/kg/hr. b. Same subject. Infusion rate = 90 mg/kg/hr for 8 hrs and 15 mins; total volume = 2 liters. c. Same subject. Infusion rate = 90 mg/kg/hr for 24 hrs; total volume = 6 liters.

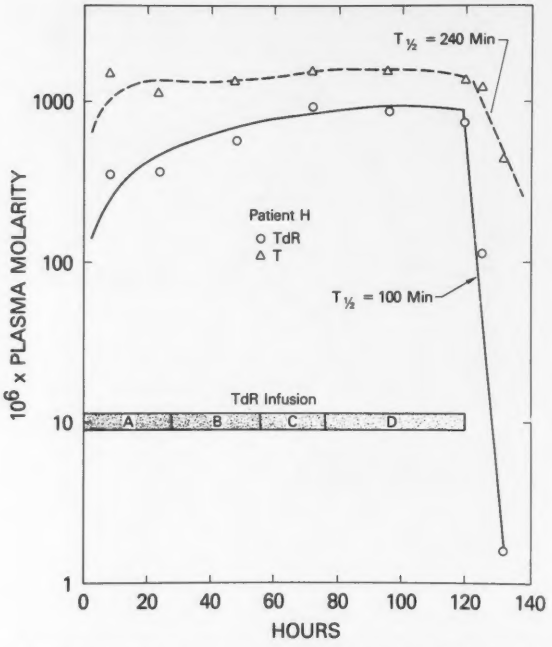

FIGURE 2.—Infusion of TdR iv into a cancer patient (80 kg). Rates of infusion: A, 47 mg/kg/hr; B, 60 mg/kg/hr; C, 75 mg/kg/hr; D, 94 mg/kg/hr. Infusion solution = 30 mg/ml of TdR in 0.6% saline.

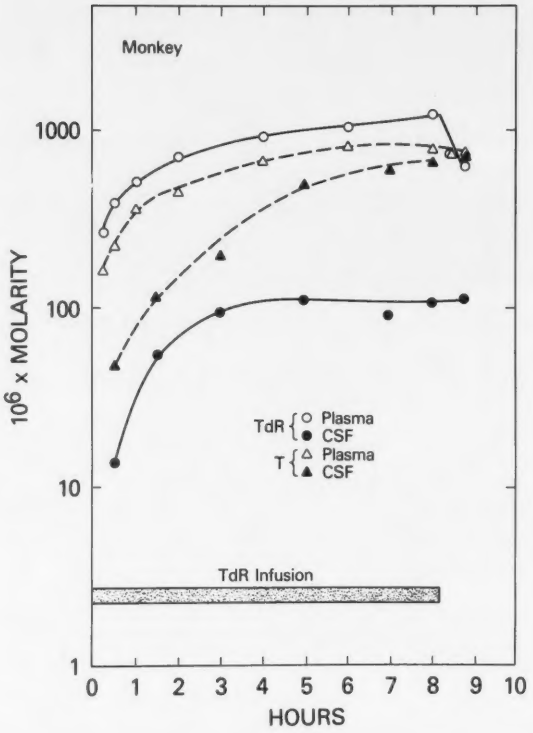

FIGURE 3.—Infusion of TdR iv into a rhesus monkey (4.8 kg). Rate of infusion = 240 mg/kg/hr. Infusion solution = 30 mg/ml of TdR in 0.6% saline. CSF samples were obtained from an indwelling needle in the cervical vertebra region.

With a constant infusion of 0.563 ml/minute of a 0.140 M TdR solution, input equals 0.075 mmols/minute. From figure 3 the average steady-state plasma concentration of TdR is 0.0012 mmols/ml. The total-body clearance is 62.5 ml/minute. Table 2 indicates that the average kidney clearance is 42.9 ml/minute for TdR and 11.1 ml/minute for T. Therefore, the kidney removes 70% of intact TdR from the circulation, another 18% is removed by the kidney as the first metabolite T, and the remaining 12% is metabolized further or removed as other metabolites by the kidney. Note that kidney clearance of TdR is four times as great as kidney clearance of T.

TABLE 2.—TdR and T kidney clearance rates during constant infusion of TdR to a monkey (0.2 g/kg/hr)*

Time interval of urine collection	Vol (ml/min)	Clearance (ml/min)	
		TdR	T
3–5	0.14	47.5	11.4
5–7	0.16	41.8	10.5
7–8	0.16	39.5	11.3

*TdR total-body clearance rate = 62.5 ml/min.

DISCUSSION

The concentration of TdR attained in these studies in the plasma of man is 10^4 times that endogenously present, and in mice the concentration is 10^3 times normal (16,17). No acute signs of toxicity were apparent in any of the animals or human subjects. However, we have seen acute lethal toxicity in mice if 10 mM TdR is maintained in plasma for as short as 20 hours. Whether chronic toxicity will occur or beneficial antitumor action will result in patients as was seen in vitro and in experimental animals at these millimolar concentrations remains to be seen. Several clinical studies are currently being conducted to determine these matters. Such supranormal concentrations have been shown to alter biochemical metabolic control processes in certain cell lines in vitro (2,18). These concentrations might also be expected to alter processes by which TdR is normally removed from the body. Earlier investigations indicate that after short- or long-term infusions when plasma TdR was < 20 μM, the half-life was about 10 minutes and < 2% of the dose appeared in the urine (9). Such data suggest that metabolic processes account for 98% of total-body clearance of TdR. Total-

body clearance of TdR in such studies at a plasma concentration of TdR of 1.5 μM calculates to be approximately 24,000 ml/minute/subject. When this is compared to an average plasma flow through the kidneys of approximately 650 ml/minute/subject, one can see that < 3% can be cleared by the kidney at this concentration of 1.5 μM. However, data collected in our investigations indicate that at plasma concentrations of millimolar TdR, metabolic processes diminish in relative importance and the kidney dominates in clearance of TdR from the body. This is indicated in man by a large reduction in total-body clearance (total-body clearance was 244 and 574 ml/minute in two subjects) and by an increase in the half-life from 10 minutes at low plasma concentrations to 80–100 minutes at high plasma concentrations.

Clearance calculations in the monkey also indicate that at these high plasma concentrations, kidney clearance predominates as the primary mechanism for TdR removal, most likely because metabolic removal processes become saturated.

At 1.5 μM TdR in plasma during TdR infusion in man (9) the CSF concentration of TdR was equal to that of the plasma after 2 hours. In contrast, at 1.2 mM TdR in plasma during TdR infusion in the monkey, equilibrium of CSF is reached after 2–3 hours, but at a CSF concentration of TdR ten times lower than that of plasma. This suggests some saturable transport process from plasma to CSF for TdR. T, on the other hand, appears to slowly enter the CSF from the plasma so that concentrations in both fluids become equal by 8 hours. Recent spot checks in several patients indicated CSF/plasma ratios of 0.15–0.35 for TdR and 0.94–1.13 for T at steady-state plasma concentrations in the millimolar range.

Further detailed studies of TdR and T in plasma, urine, and CSF are being carried out on patients receiving several courses of TdR infusions and will be reported later.

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Effect of a Forced Diuresis on the Distribution and Excretion (Via Urine and Bile) of ^{195m}Platinum When Given as ^{195m}Platinum *cis*-Dichlorodiammineplatinum(II) [1,2]

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SUMMARY

^{195m}Platinum *cis*-dichlorodiammineplatinum(II) (^{195m}Pt *cis*-DDP) was injected iv with furosemide or mannitol into male Sprague-Dawley rats. The effect of such a diuresis on the distribution, excretion, and retention of *cis*-DDP was measured. The mannitol-treated animals had a significant increase in the urinary excretion of ^{195m}Pt over control animals for the first five 15-minute collection times and for the 24-hour cumulative urine for the 0.01-mg/kg dose. At the 6-mg/kg dose, only the first three 15-minute and 24-hour cumulative urine collections were significant. This increased excretion, however, did not affect tissue or blood concentrations of this agent. Furosemide did not affect the excretion of ^{195m}Pt. A biliary excretion of ^{195m}Pt given as ^{195m}Pt *cis*-DDP is documented. The excretion is biphasic, with a rapid phase followed by a slower protracted phase. Forced diuresis, if it affords renal protection, does so during the first 90 minutes of urinary excretion.

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cis-Dichlorodiammineplatinum(II) (*cis*-DDP), a new antineoplastic agent, produces acute renal tubular damage as its major dose-limiting toxicity. Recent human studies (1–3) have shown that a forced diuresis, using either furosemide or mannitol, has modified this nephrotoxicity. Two animal models (4,5) have shown that such a forced diuresis did ameliorate the renal toxicity using renal function changes as a parameter (BUN and creatinine). This study was undertaken to measure, during the first 24 hours, the effect of such a diuresis on the distribution, excretion, and/or retention of iv injections of radiolabeled *cis*-DDP. It is not an amelioration of toxicity study.

MATERIALS AND METHODS

Healthy male Sprague-Dawley rats were obtained 1 week prior to use and housed five to a cage in polycarbonate cages with automatic watering. The animals were fed Ralston Purina micromixed laboratory chow. After 1 week, animals weighing 300 ± 25 g were selected for study.

Radiolabeled *cis*-DDP was synthesized using the radionuclide ^{195m}platinum (^{195m}Pt) supplied by Dr. Kenneth Poggenburg of the Oak Ridge National Laboratory, Tenn. The synthesis used was a modification of Kauffman and Cowan (6), Dahra (7), and Hoeschel.[5] Only final products with a calculated purity from an absorption spectrum of *cis*-DDP of > 2.5 were used in each experiment. Purity is defined as the ratio of absorbance peak at 301 nm to the trough at 246 nm for *cis*-DDP. This is measured on a Beckman model 25 uv and visible spectrometer.

The radiolabeled product ^{195m}Pt *cis*-DDP was diluted to the desired concentrations with cold *cis*-DDP (supplied by Dr. John Penta, Investigational Drug Branch, Cancer Treatment Evaluation Program, Division of Cancer

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Treatment, National Cancer Institute, Bethesda, Md). The dose used in the following experiment delivered 10 microcuries of ^{195m}Pt *cis*-DDP/animal. The time relationship of *cis*-DDP to the diuretic used was chosen to follow as closely as possible that used in human studies. Furosemide was given to the animals at a dose of 25 mg/kg iv either 30 minutes prior to or immediately following the injections of ^{195m}Pt *cis*-DDP. Mannitol obtained at a 25% aqueous solution was diluted to a 5% solution with sterile 0.85% NaCl and infused immediately at a rate of 0.388 ml/minute. The amount of mannitol delivered was equivalent to 5% of the animal's body weight. This was infused over 20 minutes.

The animals were anesthetized with amobarbital given ip at a dose of 100 mg/kg for periods of up to 4 hours. After the initial anesthesia, an iv catheter (PE 50 polyethylene tubing) was inserted and secured into one of the external jugular veins. All medications and maintenance fluids were injected into this catheter. Maintenance fluids were delivered at a rate of 2.33 ml/hour by a Harvard Infusion/Withdrawal Multispeed pump. To facilitate a quantitative recovery of urine, a similar-sized polyethylene tubing was also inserted into the urinary bladder via an abdominal incision and sutured in place. A 60-cm length contains 0.04 ml of urine. To ensure accurate urine collection the external urethra was loosely tied.

In the animal studies measuring bile flow, PE 10 polyethylene tubing was inserted into the bile duct through a right subcostal incision.

Analysis of experimental data was performed using Dunnett's t statistics for comparing several treatments with a control. Statistical significance was shown as $P < 0.05$ (8).

Forced Diuresis Experiments

Four groups consisting of 15 animals each were used: group A, ^{195m}Pt *cis*-DDP control; group B, ^{195m}Pt *cis*-DDP plus simultaneous furosemide; group C, ^{195m}Pt *cis*-DDP plus furosemide given 30 minutes before; and group D, ^{195m}Pt *cis*-DDP plus mannitol. Five animals in each group received *cis*-DDP at levels of 0.01, 4.0, or 6.0 mg/kg of body weight. These 4- and 6-mg/kg doses are known to cause renal damage that appears at different times in our laboratory, and were chosen to evaluate the effects of a toxic dose compared to those of a trace dose on distribution, excretion, and retention. Maintenance infusions of saline for group A were set at 2.33 ml/hour for the entire 3-hour period. Saline infusions for groups B and C were started at a rate of 6.60 ml/hour for the first hour and then reduced to 2.33 ml/hour for the remaining 2.5 hours. Infusions for group D were mannitol at a rate of 0.388 ml/minute for approximately 20 minutes depending on the animal's body weight and then saline at a rate of 2.33 ml/hour for the remainder of the collection. Urine collections were started with the injections of ^{195m}Pt *cis*-DDP, and were collected in serial 15-minute samples for a total of 3 hours.

Twenty-four hours after the initial injection of ^{195m}Pt *cis*-DDP, the animals were killed with an amobarbital overdose. Twenty-four-hour cumulative urine volumes were measured (± 0.01 ml). Blood, 24-hour feces, heart, lung, liver, spleen, kidneys, testicles, bladder without urine, adrenals, prostate, skin, femur, tongue, foot pad, duodenum, ileum, colon, and parotids were weighed (± 0.01 mg). All samples were assayed for radioactivity in a Packard Auto Gamma scintillation spectrometer to determine distribution of ^{195m}Pt given as ^{195m}Pt *cis*-DDP. The counting efficiency rate of this instrument for ^{195m}Pt was 87.3%; windows were set at 56–104 kev.

Initial radioactivity of ^{195m}Pt *cis*-DDP administered to each animal was determined with a Squibb CRC-6A radioisotope calibrator set at 205.5. Animals receiving 0.01-mg/kg doses received only ^{195m}Pt *cis*-DDP; animals receiving 4.0- and 6.0-mg/kg doses received the ^{195m}Pt *cis*-DDP diluted with freshly reconstituted nonradioactive *cis*-DDP supplied for animal investigation by the National Cancer Institute.

Bile Excretion Experiment

The animals used in the bile excretion studies were housed in restraining cages with access to water and food for 24 hours. In these experiments, three groups of three animals each were studied: *cis*-DDP alone, *cis*-DDP plus simultaneous furosemide, and *cis*-DDP plus mannitol. All animals were injected with an arbitrary dose of 2 mg/kg of body weight of ^{195m}Pt *cis*-DDP. Animals were infused with saline at a rate of 0.23 ml/hour overnight in addition to the initial infusions.

RESULTS

Urinary Excretion

The diuresis induced by furosemide and mannitol was rapid in onset (< 5 minutes) and lasted approximately 90 minutes. The mean volume of urine produced is shown in table 1. The diuretic treatment groups had a fourfold to sixfold increase in urine volume over the control group. The urine volume was not affected by the dose of *cis*-DDP given. Table 2 shows the percent urinary excretion of ^{195m}Pt in relation to the amount of urine recovered for each animal in the control group for the first 90 minutes when given a dose of 0.01 mg/kg. It should be noted that the amount of ^{195m}Pt recovered was not related to the amount of urine produced. Table 3 shows that the peak of urinary excretion occurs during the first 15 minutes for the diuretic groups but not for the control group. It is again shown that the peak excretion of ^{195m}Pt is not

TABLE 1.—Mean urine volume at 90 mins for the treatment groups

Treatment group	*cis*-DDP (mg/kg)	Mean urine volume (ml ± 1 SD)
Group A, control	0.01	2.85 ± 1.36
	4	2.95 ± 1.03
	6	2.91 ± 1.54
Group B, simultaneous furosemide	0.01	12.25 ± 3.58
	4	12.98 ± 6.01
	6	12.13 ± 4.74
Group C, furosemide given 30 mins before	0.01	8.63 ± 1.18
	4	8.41 ± 1.21
	6	9.65 ± 2.09
Group D, mannitol	0.01	15.67 ± 1.86
	4	14.67 ± 5.41
	6	12.60 ± 0.46

TABLE 2.—% of initial dose of ^{195m}Pt given as ^{195m}Pt *cis*-DDP recovered in urine and urine volumes/ml for the control group at a dose of 0.01 mg/kg

Animal No.	Time in mins						Totals
	15	30	45	60	75	90	
1							
% dose	2.7	1.1	8.1	3.0	1.6	0.9	28.2
Volume	0.45	0.5	0.73	0.76	0.68	0.62	3.74
2							
% dose	0.0	10	10.5	8.5	4.4	2.1	36.0
Volume	0.18	0.11	0.10	0.10	0.18	0.41	1.08
3							
% dose	0.0	0.0	2.0	11.9	3.9	6.3	24.1
Volume	0.6	0.4	0.4	0.6	0.2	0.3	2.5
4							
% dose	0.0	0.2	9.7	6.6	5.5	1.4	23.4
Volume	1.0	1.0	0.5	0.6	0.6	0.4	4.1

TABLE 3.—Comparison of the mean % of initial dose of ^{195m}Pt given as ^{195m}Pt *cis*-DDP ± 1 SD and the mean urine volumes/ml ± 1 SD for the treatment groups

Treatment group	Time in mins					
	0–15	15–30	30–45	45–60	60–75	75–90
^{195m}Pt *cis*-DDP, control						
% dose	0.68 ± 1.35	5.65 ± 6.43	7.58 ± 3.85	7.50 ± 3.72	3.85 ± 1.64	2.68 ± 2.47
Volume	0.56 ± 34	0.50 ± 37	0.43 ± 0.26	0.52 ± 29	0.42 ± 0.26	0.43 ± 0.13
^{195m}Pt *cis*-DDP + simultaneous furosemide						
% dose	10.45 ± 3.07	3.63 ± 0.93	1.93 ± 0.30	1.35 ± 0.77	0.75 ± 32	0.43 ± 0.15
Volume	3.80 ± 0.82	2.23 ± 1.23	1.90 ± 1.04	1.77 ± 0.62	1.56 ± 0.27	0.99 ± 0.37
^{195m}Pt *cis*-DDP + furosemide given 30 mins before						
% dose	12.97 ± 4.32	8.43 ± 0.12	3.67 ± 0.21	1.50 ± 0.0	0.80 ± 0.26	0.43 ± 0.15
Volume	2.16 ± 0.70	1.97 ± 0.44	1.83 ± 0.24	1.15 ± 0.10	0.86 ± 0.24	0.67 ± 0.27
^{195m}Pt *cis*-DDP + mannitol						
% dose	**25.70 ± 1.88**	**7.55 ± 1.01**	**3.48 ± 0.41**	**1.78 ± 0.29**	**1.13 ± 0.25**	**0.55 ± 0.31**
Volume	1.75 ± 0.31	4.25 ± 0.62	4.98 ± 0.76	2.63 ± 0.59	1.33 ± 0.32	0.75 ± 0.24

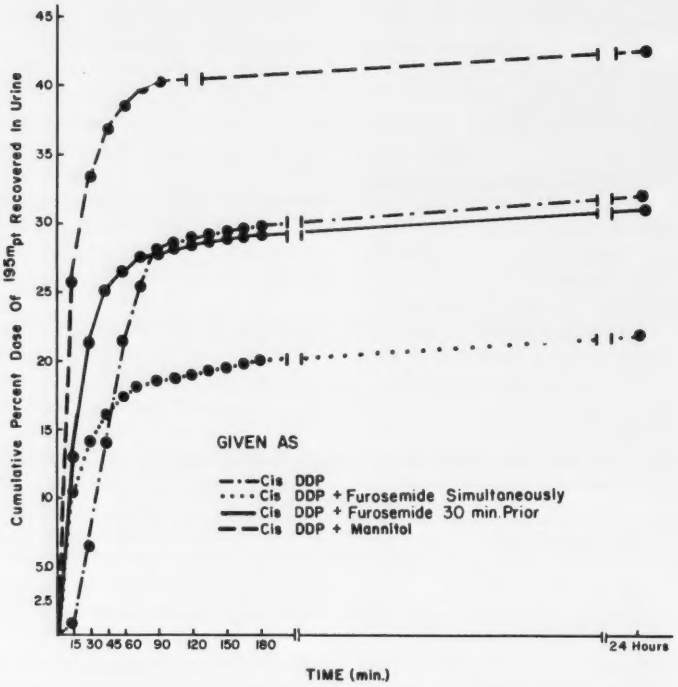

FIGURE 1.—Cumulative urinary excretion of ^{195m}Pt (0.01 mg/kg of ^{195m}Pt *cis*-DDP). Standard deviations are not shown. The maximum deviation and time it occurred for each group are as follows: group A, 45 mins ± 9.75%; group B, 24 hrs ± 5.33%; group C, 24 hrs ± 5.95%; group D, 90 mins ± 3.30%.

related to maximum urine output. The excretion curve of ^{195m}Pt given as ^{195m}Pt *cis*-DDP is similar to that previously reported in animals and man (9,10). There is a rapid initial excretion phase that terminated at 90 minutes after injection followed by a slower protracted excretion phase. Radioactivity could be detected in the urine as early as 1 minute after injection. During the rapid-excretion phase, up to 90% of the first 24-hour urinary excretion of ^{195m}Pt is recovered. Figures 1–3 show the cumulative urinary percent excretion of ^{195m}Pt given as ^{195m}Pt *cis*-DDP during the first 24 hours for the 0.01-, 4-, and 6-mg/kg doses when injected iv in the control or the diuretic groups. Figures 1 and 3 show that animals treated with mannitol had a significant increase in the percent radiolabeled platinum recovered in the urine compared to that of the control group at the 0.01- and 6-mg/kg dose. The points of significance were at 15, 30, and 45 minutes on the cumulative 24-hour collections. The 0.01-mg/kg dose had significance at the 60- and 75-minute point. The simultaneous-furosemide group had a lower mean percent excretion of ^{195m}Pt given as ^{195m}Pt *cis*-DDP when compared to that of the control group, but the difference was not significant at any of the dose levels. This is similar to the findings of Ward et al (4). The percent of ^{195m}Pt excreted for the group given furosemide 30 minutes before was identical in all of the figures to that of the control group. Therefore, it appears that furosemide given either simultaneously or prior to *cis*-DDP does not affect the first 24-hour excretion at any time point chosen. It should be noted that the overall shape of the excretion curve was not affected by the diuretics used.

If renal protection is afforded by a forced diuresis, it is apparently not done by greatly changing the 24-hour excretion of *cis*-DDP. As noted, up to 90% of the first 24-hour excretion of ^{195m}Pt *cis*-DDP occurs during the first 90-minute urine collection. Therefore, do the diuretics affect the rate of excretion of radiolabeled *cis*-DDP during this initial rapid excretion phase?

Figures 4A–C represent the rate of excretion of ^{195m}Pt during this initial phase for the different doses of the agent. As seen in all three sections of this figure, the animals treated with diuretics had a rate of excretion of the radiolabeled drug that was exponential while the control group tended to be nonexponential. As the dose was increased in the control group the excretion tended to be more and more predictable. It should be noted that the

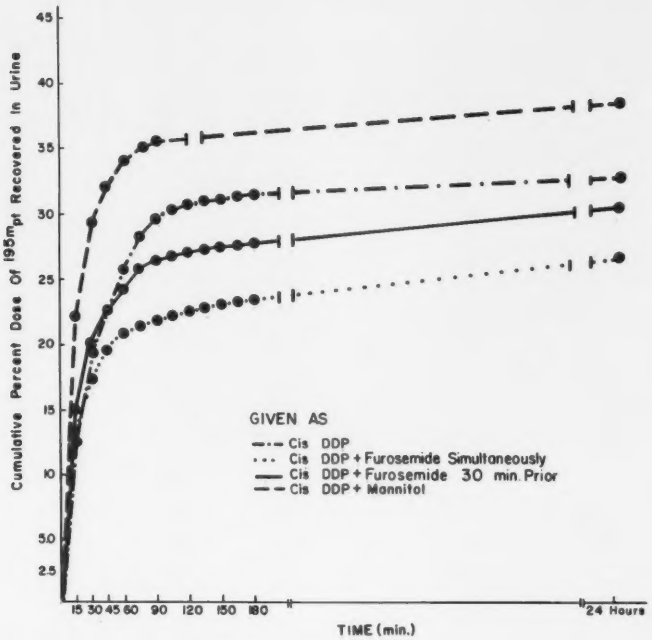

FIGURE 2.—Cumulative urinary excretion of ^{195m}Pt (4.0 mg/kg of ^{195m}Pt *cis*-DDP). Standard deviations are not shown. The maximum deviation and time it occurred for each group are as follows: group A, 45 mins ± 11.15%; group B, 24 hrs ± 9.38%; group C, 24 hrs ± 6.9%; group D, 24 hrs ± 6.65%.

timing of the furosemide did not affect the shape of the curve. One would expect the group given furosemide 30 minutes before to appear the same as the control group, much the same as it did in figures 1–3. This finding implies that the assumed protection afforded by a forced diuresis occurs early during the first 90 minutes following an iv injection.

Distribution

Table 4 represents the percent relative concentration (11) of ^{195m}Pt recovered in selected tissues that showed a significant difference for the diuretic treatment group compared to the control group. In general, however, the use of diuretic treatment did not significantly alter the concentration of ^{195m}Pt in any of the tissues. In the group given furosemide 30 minutes before, there is a significant increase in the amount of ^{195m}Pt *cis*-DDP recovered in the kidneys, blood, spleen, and questionably, the liver. This is not seen in the simultaneous-furosemide group.

Biliary Excretion

Figure 5 represents the biliary excretion curves of ^{195m}Pt given at an arbitrary dose of 2 mg/kg. Radioactivity was detected in all animal bile during the first minute of flow. The bile excretion curves were very similar to the urinary excretion curves. There is an initial rapid excretion phase followed by a slower protracted phase. As expected, neither the total biliary excretion nor the rate of excretion of ^{195m}Pt was affected by the diuretics used and was therefore not an explanation for renal protection. Table 5 shows the total percent of the initial dose recovered in the bile and urine of the three groups. Table 5 also compares the percent relative concentration of ^{195m}Pt in the feces of a control animal receiving 0.01 mg/kg of ^{195m}Pt *cis*-DDP without bile diversion to that of an animal receiving 2 mg/kg of ^{195m}Pt *cis*-DDP with bile diversion. This is a valid comparison. Table 4 shows that as the initial dose of *cis*-DDP is increased from 0.01 to 4 mg/kg, the percent concentration in the feces remains constant. Bile diversion causes a sixfold decrease of ^{195m}Pt recovered in the feces. Table 5 also shows that urinary excretion of ^{195m}Pt is unchanged when bile flow is diverted during the first 24 hours.

COMMENTS

Several studies have suggested that a forced diuresis can protect a patient or animal from renal damage caused by *cis*-DDP (1–5). The diuretics used in these studies were furosemide and mannitol given with liberal amounts

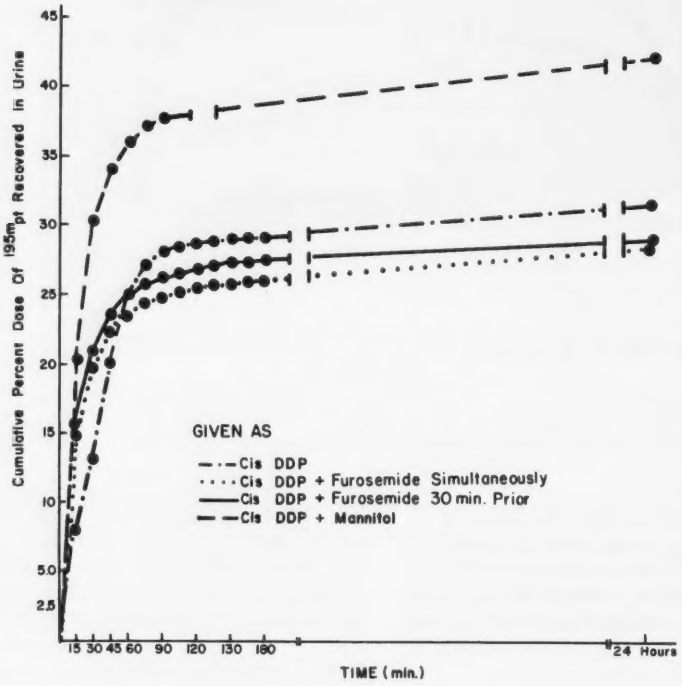

FIGURE 3.—Cumulative urinary excretion of ^{195m}Pt (6.0 mg/kg of ^{195m}Pt *cis*-DDP). Standard deviations are not shown. The maximum deviation and time it occurred for each group are as follows: group A, 30 mins ± 12.27%; group B, 24 hrs ± 5.96%; group C, 60 mins ± 5.92%; group D, 15 mins ± 6.06%.

TABLE 4.—Relative concentration* of ^{195m}Pt given as ^{195m}Pt *cis*-DDP in selected tissues 24 hrs after injection

Dose (mg/kg iv)	Treatment group					
	cis-DDP† (control)	*cis*-DDP + simultaneous furosemide†		*cis*-DDP + furosemide given 30 mins before†		*cis*-DDP + mannitol†	
Kidneys							
0.01	1.98 ± 0.30	2.82 ± 1.25		3.87 ± 0.58	($P < 0.01$)‡	3.05 ± 0.28	($P < 0.01$)‡
4.0	2.08 ± 0.53	3.23 ± 1.26		3.45 ± 0.76	($P < 0.02$)‡	2.31 ± 0.37	
6.0	2.08 ± 0.44	2.67 ± 0.38		3.41 ± 1.10	($P < 0.05$)	2.08 ± 0.13	
Blood							
0.01	0.22 ± 0.03	0.31 ± 0.08		0.58 ± 0.13	($P < 0.01$)‡	0.32 ± 0.02	($P < 0.01$)‡
4.0	0.23 ± 0.05	0.30 ± 0.98		0.40 ± 0.09	($P < 0.02$)‡	0.24 ± 0.02	
6.0	0.23 ± 0.04	0.28 ± 0.05		0.35 ± 0.12		0.25 ± 0.01	
Lungs							
0.01	0.26 ± 0.03	0.44 ± 0.08	($P < 0.01$)‡	0.52 ± 0.16	($P < 0.05$)‡		
4.0	0.34 ± 0.13	0.39 ± 0.18		0.61 ± 0.34		ND§	
6.0	0.39 ± 0.19	0.38 ± 0.12		0.48 ± 0.14			
Spleen							
0.01	0.21 ± 0.03	0.28 ± 0.08		0.56 ± 0.37			
4.0	0.28 ± 0.05	0.37 ± 0.06		0.52 ± 0.04	($P < 0.0001$)‡	ND§	
6.0	0.27 ± 0.02	0.32 ± 0.07		0.40 ± 0.10	($P < 0.05$)‡		
Liver							
0.01	0.43 ± 0.05	0.56 ± 0.24		0.76 ± 0.15	($P < 0.01$)‡	0.61 ± 0.06	($P < 0.01$)‡
4.0	0.51 ± 0.15	0.60 ± 0.15		0.67 ± 0.12		0.55 ± 0.03	
6.0	0.50 ± 0.13	0.55 ± 0.11		0.65 ± 0.18		0.56 ± 0.03	
Skin							
0.01	0.83 ± 0.65	0.39 ± 0.03		ND§		0.71 ± 0.02	
4.0	0.47 ± 0.09	0.61 ± 0.06		0.69 ± 0.02	($P < 0.05$)‡	0.61 ± 0.17	
6.0	0.48 ± 0.17	0.70 ± 0.03		0.72 ± 0.02		0.53 ± 0.04	
Tongue							
0.01	0.15 ± 0.005	0.16 ± 0.02		ND§			
4.0	0.17 ± 0.003	0.27 ± 0.13		0.47 ± 0.05	($P < 0.002$)‡	ND§	
6.0	0.18 ± 0.004	0.40 ± 0.009	($P < 0.0002$)‡	0.44 ± 0.21			
Feces							
0.01	1.74 ± 1.09	3.19 ± 1.67		4.59 ± 4.02		4.87 ± 1.50	
4.0	1.80 ± 0.79	2.13 ± 1.98		3.55 ± 1.75		2.86 ± 1.32	
6.0	4.03 ± 2.29	4.23 ± 1.43		2.47 ± 1.84		4.81 ± 0.98	

$$\text{*Relative concentration} = \frac{\text{activity (microcuries) found per g of specimen}}{\text{activity (microcuries) administered per g of body weight}}.$$

†Mean ± 1 SD.

‡Significant compared to control group.

§No determinations.

TABLE 5.—Mean % excretion of ^{195m}Pt given as 2 mg/kg of *cis*-DDP with biliary flow diversion in urine, bile, and feces at 24 hrs

	Treatment group		
	cis-DDP (control)	*cis*-DDP + furosemide	*cis*-DDP + mannitol
Mean % of initial dose (± 1 SD)			
Urine	34.7 ± 7.5	34.9 ± 6.3	42.8 ± 1.8
Bile	1.42 ± 0.66	1.62 ± 0.72	2.04 ± 0.09
Mean relative concentration (± 1 SD)			
Feces	0.281 ± 0.09	0.171 ± 0.10	0.432 ± 0.05
Mean relative concentration in feces for 0.01 mg/kg of *cis*-DDP without bile diversion	1.74 ± 1.09	3.19 ± 1.67	4.87 ± 1.50

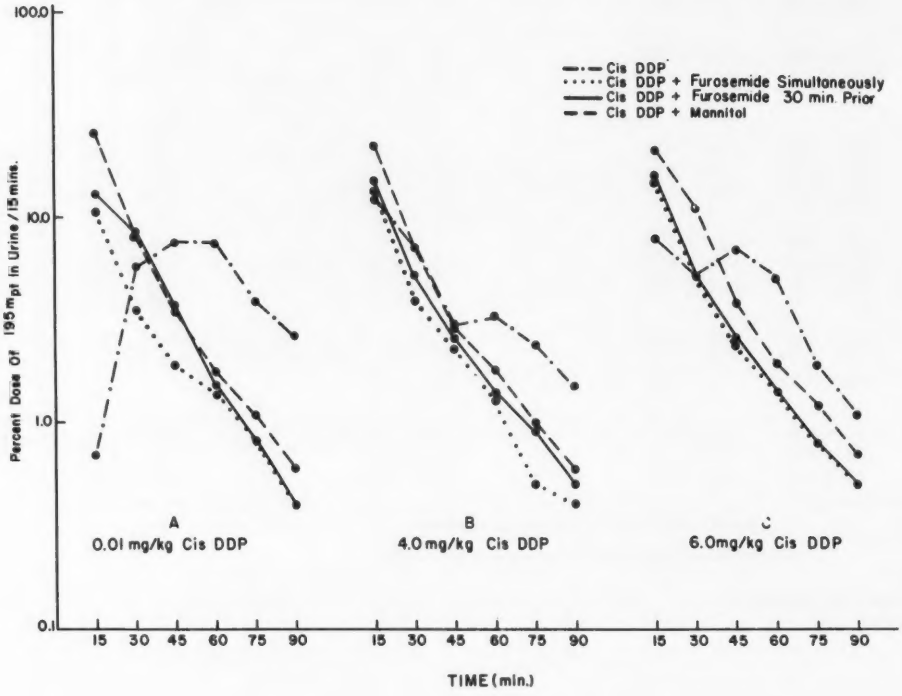

FIGURE 4.—Urinary excretion of ^{196m}Pt.

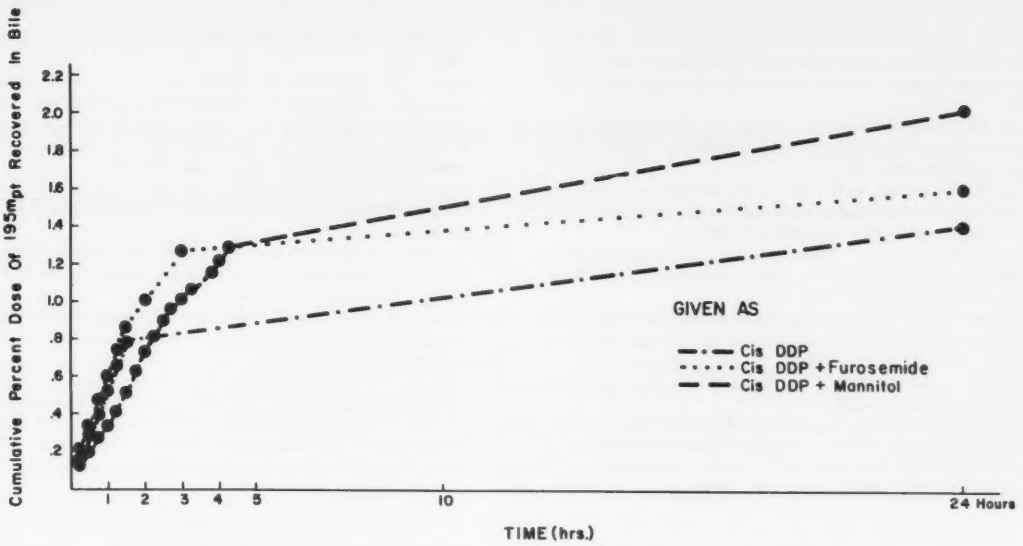

FIGURE 5.—Biliary excretion of ^{195m}Pt (2 mg/kg of ^{195m}Pt *cis*-DDP).

of iv fluids. Human studies (1–3) noted that the dose of *cis*-DDP could be increased from 1 to 3 mg/kg with equivalent renal toxicity (as measured by changes in BUN, creatinine, and creatinine clearance) if diuresis is given at the increased dose level. The studies in dogs and Fischer 344 rats showed similar results. Despite these observations, the mechanism of the protective effect is unknown. Several investigators (12) have reported that there is histologic evidence of renal damage even though a forced diuresis was used. No data have been published concerning the effects of a forced diuresis on urinary excretion or tissue distribution of *cis*-DDP. This study is the first to report the distribution and excretion patterns of ^{195m}Pt when given as ^{195m}Pt *cis*-DDP with a variety of diuretics.

Mannitol caused an increased urinary excretion of ^{195m}Pt which would be expected to influence therapeutic effectiveness of the drug. This increased renal loss was, however, not reflected as a decrease in blood or tissue concentrations, and therefore, it might be assumed that it did not affect the therapeutic effectiveness. In figures 1–3 the cumulative excretion curves always appear in the same order no matter what the dose of *cis*-DDP. The mean excretion for the control and the furosemide groups was approximately 30% of the initial dosage. The relative concentration of ^{195m}Pt recovered in the tissues of the control group was also constant for a specific tissue. This implies that the body may handle the *cis*-DDP in exactly the same way regardless of the amount given.

Biliary excretion of *cis*-DDP had been implied in human distribution studies (13–15). The only animal study (9) that was performed used dogs. Very small amounts of *cis*-DDP were detected in the bile for time periods of up to 12 days. From the data described above, roughly 1%–2% of a given dose of ^{195m}Pt given as ^{195m}Pt *cis*-DDP will be excreted in the bile during the first 24 hours; its biliary excretion is not affected by the use of diuretics. This study and the data in the literature imply that there may be a difference in the way *cis*-DDP or its metabolites are excreted in the animal model chosen. Even though there are no direct comparative data for equivalent doses of *cis*-DDP, the amount of radiolabel recovered in the feces comes from biliary excretion. The question of whether some of this biliary ^{195m}Pt is re-absorbed from the small bowel is not known.

CONCLUSIONS

Mannitol diuresis causes an increased urinary excretion of ^{195m}Pt without changing the relative concentration in the tissues. The linear excretion of ^{195m}Pt with diuretics may be the means by which renal protection is afforded. There is, at least in the rat, a biliary excretion of ^{195m}Pt which accounts for up to 2% of the excretion of ^{195m}Pt during the first 24 hours.

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Biologic Activity of MCNU: A New Antitumor Agent[1]

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SUMMARY

A novel nitrosourea derivative, methyl-6-[[[(2-chloroethyl)nitrosoamino]carbonyl]-amino]-6-deoxy-α-D-glucopyranoside (MCNU), is a water-soluble compound in which a methoxyl group is attached to the C-1 position and an *N*-(2-chloroethyl)-*N*-nitrosoureido group is attached to the C-6 position of the glucose moiety. MCNU exhibited a marked life-prolongation or growth-inhibitory effect against mouse L1210 leukemia, adenocarcinoma 755, Nakahara-Fukuoka sarcoma, Lewis lung carcinoma, and B16 melanoma. Ip, oral, or iv administration of MCNU was markedly effective against L1210 leukemia, and the therapeutic ratio by ip administration was larger than that of chlorozotocin or CCNU. The life-prolongation effect of MCNU against established Lewis lung carcinoma was similar to that of methyl-CCNU. The bone marrow toxicity of MCNU was less than that of CCNU but considerably more than that of chlorozotocin.

[Cancer Treat Rep 63:961–970, 1979]

Although the lipophilic nitrosourea derivatives BCNU,[4] CCNU,[5] and methyl-CCNU[6] have been tested for their clinical efficacies, hydrophilic derivatives such as 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) (1), chlorozotocin (2), and 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea (GANU) (3) have been reported to be effective in animal tumors when given by iv administration, possessing only a mild myelosuppressive effect (2–5).

Nitrosourea compounds related to streptozotocin[7] were synthesized in our laboratories and we examined their biologic activities. Among the compounds synthesized, methyl-2-deoxy-2-(3-methyl-3-nitrosoureido)-β-D-glucopyranoside (6) and 1-(2-chloroethyl)-3-(methyl-α-D-glucopyranos-2-yl)-1-nitrosourea (7) were found to possess marked antitumor activity, showing no diabetogenic effect as streptozotocin did. Following these observations, we also found and reported (8) that methyl-6-[[[(2-chloroethyl)nitrosoamino]carbonyl]amino]-6-deoxy-α-D-glucopyranoside (MCNU; fig 1), a water-soluble nitrosourea derivative, was markedly effective against L1210 leukemia.

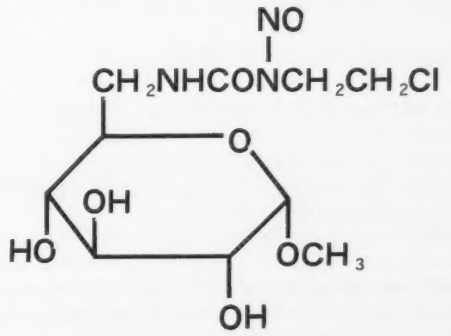

FIGURE 1.—Structure of MCNU.

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[3]We are greatly indebted to Drs. Yoshio Sakurai, Shigeru Tsukagoshi, and Tazuko Tashiro (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research) for their kind advice and cooperation in this study.

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In this paper, we describe the antitumor effect of MCNU against various animal tumors and the effect of MCNU on peripheral wbc counts.

MATERIALS AND METHODS

Animals

Female hybrid (C57BL/6 × DBA/2)F_1 ($BD2F_1$) mice, weighing 20–22 g, 8–9-week-old male DBA/2 mice, and 10-week-old male C57BL/6 mice were supplied by Charles River Japan Inc (Atsugi, Japan). ICR-JCL female mice (5 weeks old) were supplied by CLEA Japan Inc (Tokyo). All animals were maintained at a room temperature of 23°C ± 1°C and a relative humidity of 60% ± 5%.

Tumors

For the evaluation of the antitumor activity of MCNU, L1210 leukemia, adenocarcinoma 755, Nakahara-Fukuoka sarcoma, Lewis lung carcinoma, and B16 melanoma were used.

Ascitic L1210 leukemia supplied by the Cancer Chemotherapy Center, Tokyo, Japan (originally received from Mr. I. Wodinsky, Arthur D. Little, Inc, Cambridge, Mass, every 3 months) was maintained by serial passage in DBA/2 mice and transplanted by ip inoculation into $BD2F_1$ mice (1×10^5 cells/mouse). Adenocarcinoma 755 supplied by Dr. K. Kuretani of the National Cancer Center, Tokyo, Japan, was maintained in $BD2F_1$ mice and transplanted sc into $BD2F_1$ mice (about 30-mg tumor fragments/mouse). Lewis lung carcinoma and B16 melanoma, also supplied by the Cancer Chemotherapy Center, were maintained in C57BL/6 mice and transplanted sc into $BD2F_1$ mice (1×10^6 viable cells for Lewis lung carcinoma and 0.5 ml of tumor suspension, 1 g/10 ml, for B16 melanoma).

Nakahara-Fukuoka sarcoma was supplied by Dr. K. Kuretani of the National Cancer Center, and was maintained in ICR-JCL mice and transplanted sc into ICR-JCL mice (about 50-mg tumor fragments/mouse).

Drugs

MCNU was synthesized in our laboratory and dissolved in sterile physiologic saline before use. Doses of 0.2 ml/10 g of body weight were given ip, iv, or orally to experimental animals. Chlorozotocin was administered in the same manner as MCNU.

CCNU and methyl-CCNU were suspended in 0.5% carboxymethyl cellulose. Doses of 0.1 ml/10 g of body weight were given ip or orally. In this injection volume, 0.5% carboxymethylcellulose was not toxic when administered to experimental animals.

Antitumor Activity

Antitumor effect was examined using primarily the screening protocols of the National Cancer Institute (Bethesda, Md) (9). In L1210 leukemia, the antitumor activity was established by the increase in lifespan (ILS) and the number of 60-day survivors after single (Day 1, ip or oral), intermittent (Days 1 and 9, ip), or multiple (Days 1–5, ip or oral) treatments.

The antitumor activities in adenocarcinoma 755 and Nakahara-Fukuoka sarcoma were judged from the inhibition of tumor weight (TWI) by killing the mice 12 days after transplantation for adenocarcinoma 755 and 10 days after transplantation for Nakahara-Fukuoka sarcoma after multiple treatment (Days 1–5, ip or oral). Change in body weight was examined as an indicator of drug toxicity.

The antitumor activity in Lewis lung carcinoma and B16 melanoma was established by the ILS and the number of 60-day survivors by measuring the median survival time after early treatments (Day 1 or 2, ip), multiple treatments (Days 1–11, ip), and established treatments (Days 7 and 13, ip; Days 7, 11, 15, and 19, ip; Days 7, 11, and 15, ip; and Days 13, 17, 21, and 25, ip) for Lewis lung carcinoma, and after early treatments (Day 1, ip; Day 2, ip or oral), multiple treatments (Days 1–9, ip), established treatments (Day 11, ip or oral; and Days 13, 17, 21, and 25, ip), and intermittent treatment (Days 1, 5, 9, and 13, ip) for B16 melanoma.

For the purpose of examining the tumor growth after implantation, major and minor diameters of the tumor were measured with a caliper and the tumor weight was calculated by the following formula (10):

$$\text{Mass (in mg)} = \frac{\text{major diameter (in mm)} \times \text{minor diameter (in mm)}^2}{2}$$

Peripheral Wbc Counts

The leukopenic action of MCNU was investigated in comparison with those of chlorozotocin and CCNU after a single ip injection into each group of five (treated group) or seven (control group) female $BD2F_1$ mice. The blood was taken with a glass capillary from the retro-orbital sinus of the mice. Each group of mice was killed on Days 3, 7, 14, or 21 after injection. The wbcs were stained with Türk's solution and counted using a hemocytometer. Lethality was calculated by the number of deaths among five normal $BD2F_1$ mice for 21 days after administration. Statistical significance was determined by the Student's t test.

TABLE 1.—Antitumor activity of MCNU, chlorozotocin, and CCNU against L1210 leukemia*

Treatment	Compounds†	Dose (mg/kg/day)	Mean survival time (excluding survivors) T/C‡ (days)	%ILS	Survivors on Day 60/ total No. in group
Days 1–5, ip	MCNU	1.6	15.2/7.5	103	0/6
		3.2	13.0/7.5	73	4/6
		6.3	–/7.3	—	6/6
		12.5	–/7.3	—	6/6
		25	12.0/7.3	64	3/6
		50	8.3/7.3	14	0/6
	Chlorozotocin	6.3	16.0/7.3	119	5/6
	CCNU	18	–/7.6	—	6/6
Day 1, ip	MCNU	1.6	10.9/7.4	47	0/6
		3.2	13.4/7.4	81	1/6
		6.3	16.7/8.3	101	3/6
		12.5	–/7.3	—	6/6
		25	–/7.3	—	6/6
		35	–/8.2	—	6/6
		50	21.0/7.3	188	3/6
	Chlorozotocin	25	–/7.5	—	6/6
	CCNU	50	–/8.0	—	6/6
Days 1–5, oral	MCNU	3.2	37.3/8.0	366	0/6
		6.3	14.7/7.5	96	3/6
		12.5	–/7.5	—	6/6
		18	–/7.5	—	6/6
		25	25.0/7.5	233	0/6
	Chlorozotocin	100	9.2/7.8	18	0/6
	CCNU	35	–/8.2	—	6/6
Day 1, iv	MCNU	3.2	8.5/7.8	9	0/6
		6.3	12.0/7.8	54	1/6
		12.5	15.0/7.8	92	3/6
		25	20.0/7.8	156	5/6
		50	26.3/7.8	237	3/6
	Chlorozotocin	12.5	13.3/8.1	64	2/6
Days 1 and 9, ip	MCNU	1.6	10.7/7.5	43	0/6
		3.2	25.0/7.5	233	4/6
		6.3	20.7/7.3	184	3/6
		12.5	20.0/7.5	167	4/6
		25	–/7.3	—	6/6
		50	43.5/7.5	480	2/6

*Inoculum: 1×10^5 cells/$BD2F_1$ mouse, ip.
†MCNU and chlorozotocin were dissolved in sterile saline. CCNU was suspended in 0.5% carboxymethylcellulose solution.
‡T/C = treated/control.

RESULTS

Effect on L1210 Leukemia

The antitumor activity of MCNU, chlorozotocin, and CCNU on ascitic L1210 leukemia is shown in table 1. A marked ILS was observed by ip, oral, or iv treatment. With ip treatment, maximum effective doses were 6.3–12.5 mg/kg/day (Days 1–5), 12.5–35 mg/kg (Day 1), and 25 mg/kg/day (Days 1 and 9). In these cases, all mice survived > 60 days. With oral treatment, the optimal dose by continuous administration (Days 1–5) was 18 mg/kg/day, and all mice survived > 60 days. However, 25 mg/kg/day was toxic and there were no 60-day survivors. With iv treatment, the optimal single dose (Day 1) was 25 mg/kg; five of six mice survived > 60 days. From these results, antitumor effect by oral and iv treatments was slightly less than that by ip treatment.

The therapeutic ratios obtained from ILS_{30} and optimal dose are shown in table 2. The therapeutic ratios of MCNU in different treatment schedules were 34.7 (Days 1–5, ip), 29.2 (Day 1, ip), 25.0 (Days 1 and 9, ip), 7.2 (Days 1–5, oral), and 7.1 (Day 1, iv). On the other hand, the ratios of chlorozotocin were 9.7 (Days 1–5, ip), 13.5 (Day 1, ip), and 2.0 (Day 1, iv), revealing single ip treatment to be the best. Moreover, oral administration of

TABLE 2.—Indices of the compounds in the L1210 leukemia system*

Treatment	Compounds	Dose producing ILS_{30}† (mg/kg/day)	Optimal dose (mg/kg/day)	Survivors on Day 60/ total No. in group	Therapeutic ratio‡
Days 1–5, ip	MCNU	0.36	12.5	6/6	34.7
	Chlorozotocin	0.65	6.3	5/6	9.7
	CCNU	2.0	18.0	6/6	9.0
Day 1, ip	MCNU	1.2	35.0	6/6	29.2
	Chlorozotocin	1.85	25.0	6/6	13.5
	CCNU	6.3	50.0	6/6	7.9
Days 1–5, oral	MCNU	2.5	18.0	6/6	7.2
	Chlorozotocin	—	100.0	0/6	—
	CCNU	3.5	35.0	6/6	10.0
Day 1, iv	MCNU	3.5	25.0	5/6	7.1
	Chlorozotocin	6.3	12.5	2/6	2.0
Days 1 and 9, ip	MCNU	1.0	25.0	6/6	25.0

*Inoculum: 1×10^5 cells/$BD2F_1$ mouse, ip.
†ILS_{30} = an increase in lifespan of 30% over controls.
‡Therapeutic ratio = optimal dose/dose producing ILS_{30}.

chlorozotocin was almost ineffective and we were unable to obtain a therapeutic ratio. The ratios of CCNU were 7.9 (Day 1, ip) and 9.0 (Days 1–5, ip). There was no significant difference in the therapeutic ratio of CCNU between oral and ip multiple treatments (10.0 by oral treatment).

Effect on Adenocarcinoma 755 and Nakahara-Fukuoka Sarcoma

The antitumor activities of MCNU, chlorozotocin, and CCNU against adenocarcinoma 755 and Nakahara-Fukuoka sarcoma are shown in table 3. The percent of TWI of MCNU against adenocarcinoma 755 was > 95% by ip or oral administration of 25 mg/kg/day, and there were no toxic deaths. The antitumor activity of MCNU in Nakahara-Fukuoka sarcoma was a little less than that in adenocarcinoma 755, with 66.4% TWI (12.5 mg/kg/day, ip) and 72.7% TWI (25 mg/kg/day, oral) being obtained. CCNU showed an almost similar antitumor effect against these two tumor strains. However, chlorozotocin showed a little less activity against adenocarcinoma 755 than did MCNU and CCNU.

Effect on Lewis Lung Carcinoma

Table 4 indicates the antitumor effect of MCNU and methyl-CCNU on Lewis lung carcinoma. Growth-inhibitory and life-prolongation effects of MCNU were observed irrespective of treatment schedules (table 4). An especially marked effect was noted with early treatment (Day 1 or 2, ip). With single ip administration of MCNU (Day 1) at 20, 35, or 50 mg/kg, almost all animals were cured. With another single-treatment schedule (Day 2, ip), eight of ten mice given 35 mg/kg were cured.

With multiple treatment (Days 1–11, ip), six of ten mice were cured at a dose of 12.5 mg/kg/day.

With established treatment, in which MCNU was given from Day 7 (tumor weight, 400–500 mg) or Day 13 (tumor weight, about 1.2 g), the antitumor activity was a little less than that obtained with the early treatment. However, on the optimal treatment schedule (Days 7, 11, and 15, ip), three of ten mice were cured.

On the other hand, methyl-CCNU showed an antitumor effect similar to that of MCNU by ip treatments on Day 2 and on Days 7 and 13.

Effect on B16 Melanoma

The antitumor activity of MCNU and methyl-CCNU against B16 melanoma is shown in table 5; ip and oral administrations of MCNU were effective. The ILS at 35 mg/kg was 46% (Day 2, ip) and 76% (Day 11, ip), which are almost the same as the ILSs obtained with methyl-CCNU. With intermittent treatment with MCNU (Days 1, 5, 9, and 13, ip), the ILS at 35 mg/kg was 67%, and three of ten tumor-bearing mice survived > 60 days. This treatment schedule was found to be better than the others tested. With oral treatment on Day 2 the antitumor effect was almost the same as that obtained with Day-2 ip treatment, but with oral treatment on Day 11 there was a tendency for the optimal dose to shift to higher doses.

Effect on Peripheral Wbc Counts

Figure 2 shows the leukopenic action of MCNU compared with those of chlorozotocin and CCNU on $BD2F_1$ mice after a single ip injection. The nadir wbc count was found on Day 3 after injection for these three compounds.

TABLE 3.—Antitumor activity of MCNU, chlorozotocin, and CCNU against solid tumors*

Tumor system	Compounds†	Administration route	Dose (mg/kg/day)	%TWI	Body weight change in grams‡ (T–C)	Toxicity§
Adenocarcinoma 755	MCNU	ip	3.2	40.1	+1.2	0/6
			6.3	72.4	+2.7	0/6
			12.5	95.8	+0.5	0/6
			25	100.0	−6.4	0/6
		Oral	6.3	74.8	−1.9	0/6
			12.5	84.3	−0.4	0/6
			25	96.5	−4.4	0/6
			50	—	—	6/6
	Chlorozotocin	ip	1.6	12.3	+0.3	0/6
			3.2	44.0	−0.5	0/6
			6.3	73.9	−2.0	0/6
			12.5	—	—	6/6
	CCNU	ip	10	85.0	−2.4	0/6
			15	98.9	−6.4	2/6
Nakahara-Fukuoka sarcoma	MCNU	ip	3.2	17.2	−0.4	0/6
			6.3	53.7	−3.6	0/6
			12.5	66.4	−2.5	0/6
			25	85.7	−8.5	1/6
		Oral	6.3	27.5	−0.5	0/6
			12.5	45.9	0	0/6
			25	72.7	−7.4	0/6
			50	—	—	6/6
	Chlorozotocin	ip	1.6	15.7	−1.9	0/6
			3.2	45.7	−3.1	0/6
			6.3	50.0	−4.7	0/6
			12.5	82.4	−12.1	4/6
	CCNU	ip	12	42.5	−2.9	0/6
			20	51.7	−1.2	0/6
			25	70.4	−7.6	1/6

*Mice were given sc injections of 20–40-mg tumor fragments on Day 0. Treatment was initiated 24 hrs after implantation of tumor for 5 consecutive injections. Antitumor activity was judged from the TWI on Day 12 for adenocarcinoma 755 or on Day 10 for Nakahara-Fukuoka sarcoma.

†MCNU and chlorozotocin were dissolved in sterile saline. CCNU was suspended in 0.5% carboxymethylcellulose solution.

‡The average weight change was based on the change from Day 1 to the day of evaluation excluding tumor weight.

§No. of dead animals/total in treated group until the day of evaluation.

TABLE 4.—Antitumor activity of MCNU against Lewis lung carcinoma*

Treatment	Compounds†	Dose (mg/kg/day)	%TWI on Day 20	Median survival time (excluding survivors)		Survivors on Day 60/ total No. in group	
				T/C‡ (days)	%ILS	With tumor	Without tumor
Day 1, ip	MCNU	20	99.9	36.0/27.0	33	0/10	9/10
		35	100.0	–/27.0	—	0/10	10/10
		50	100.0	15.0/27.0	– 44	0/10	8/10
Day 2, ip	MCNU	20	76.6	35.5/28.0	27	0/9	1/9
		35	100.0	39.5/28.0	41	0/10	8/10
	Methyl-CCNU	20	100.0	–/31.0	—	0/10	10/10
		40	100.0	–/31.0	—	0/10	10/10
Days 1–11, ip	MCNU	3.2	29.2	29.5/22.0	34	0/10	0/10
		6.3	59.1	35.0/22.0	59	0/10	0/10
		12.5	100.0	18.0/22.0	– 18	0/10	6/10
Days 7 and 13, ip	MCNU	10	2.2	30.0/28.0	7	0/10	0/10
		20	55.6	22.0/28.0	– 21	0/10	0/10
		35	98.6	49.5/28.0	77	2/10	0/10
	Methyl-CCNU	10	31.1	35.5/30.5	16	0/10	0/10
		20	97.6	50.0/30.5	64	0/10	2/10
		40	96.3	25.0/30.5	– 18	0/10	2/10
Days 7, 11, 15, and 19, ip	MCNU	20	78.6	40.0/26.5	51	0/10	0/10
Days 7, 11, and 15, ip	MCNU	35	93.8	36.0/26.5	36	2/10	3/10
Days 13, 17, 21, and 25, ip	MCNU	10	3.9§	27.5/24.0	15	0/10	0/10
		20	61.1§	40.0/24.0	67	1/10	0/10
		35	84.2§	42.0/24.0	75	0/10	0/10

*Inoculum: 1×10^6 viable cells/$BD2F_1$ mouse, sc.
†MCNU was dissolved in sterile saline. Methyl-CCNU was suspended in 0.5% carboxymethylcellulose solution.
‡T/C = treated/control.
§Calculated on Day 25.

TABLE 5.—Antitumor activity of MCNU against B16 melanoma*

Treatment	Compounds†	Dose (mg/kg/day)	%TWI on Day 20	Median survival time (excluding survivors)		Survivors on Day 60/total No. in group
				T/C‡ (days)	%ILS	
Day 1, ip	MCNU	35	75.9	38.0/26.0	46	0/9
		50	94.8	22.0/26.0	− 15	0/10
Day 2, ip	MCNU	20	34.4	29.0/26.0	12	0/10
		35	83.2	38.0/26.0	46	0/10
	Methyl-CCNU	10	− 13.2	30.0/28.5	5	0/10
		20	46.7	39.0/28.5	37	0/9
		40	91.1	17.0/28.5	− 40	0/10
Day 11, ip	MCNU	20	68.3	32.0/23.0	39	0/10
		35	80.4	40.5/23.0	76	0/10
	Methyl-CCNU	10	14.4	24.0/29.0	− 17	0/9
		20	75.9	51.0/29.0	76	1/10
		40	84.5	39.0/29.0	34	1/10
Days 1–9, ip	MCNU	3.2	43.1	28.0/21.0	33	0/10
		6.3	72.5	34.0/21.0	62	0/10
		12.5	98.0	30.0/21.0	43	0/10
Days 1, 5, 9, and 13, ip	MCNU	20	98.3	50.0/30.5	64	1/10
		35	100.0	51.0/30.5	67	3/10
Days 13, 17, 21, and 25, ip	MCNU	20	68.2§	36.0/26.5	36	1/9
		35	79.3§	37.5/26.5	42	0/10
Day 2, oral	MCNU	35	81.3	42.5/30.0	42	0/10
		50	92.4	42.5/30.0	42	0/10
		70	96.1	37.5/26.0	44	0/10
Day 11, oral	MCNU	35	75.1	34.0/28.0	21	0/10
		50	75.7	37.5/28.0	34	0/10
		70	66.8	42.0/26.0	62	1/10

*Inoculum: 0.5 ml of tumor suspension (1 g/10 ml)/$BD2F_1$ mouse, sc.
†MCNU was dissolved in sterile saline. Methyl-CCNU was suspended in 0.5% carboxymethylcellulose solution.
‡T/C = treated/control.
§Calculated on Day 25.

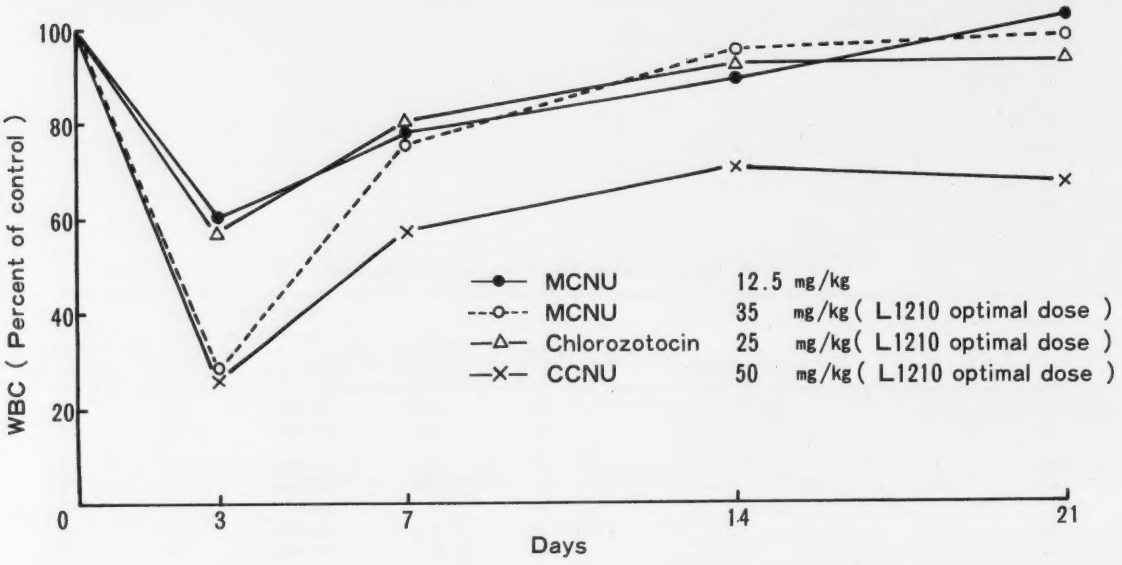

FIGURE 2.—Effect of MCNU, chlorozotocin, and CCNU on serial peripheral wbc counts in $BD2F_1$ mice.

When each compound was administered ip at the optimal dose obtained in L1210 leukemia, it was observed that the wbc count depression from MCNU or chlorozotocin recovered to the normal level after 14 days. On the other hand, the depression from CCNU lasted > 21 days, revealing 73% ($P < 0.01$), 43% ($P < 0.05$), 30% ($P < 0.05$), and 32% ($P < 0.05$) reduction of wbc counts on Days 3, 7, 14, and 21 respectively. These values were significantly different from control values.

Table 6 shows the nadir (Day 3) wbc counts and the antitumor activity in L1210 leukemia for these three compounds. MCNU administered at the optimal dose for antileukemic activity, 35 mg/kg, produced a 73% reduction in mean wbc counts, which was significantly different from control values ($P < 0.01$). A 40% reduction in wbc counts ($P < 0.01$) was produced at a dose of 12.5 mg/kg, which revealed a maximum antileukemic effect (all leukemic mice survived > 60 days). Chlorozotocin produced a 41% reduction ($P < 0.01$) at the optimal dose of 25 mg/kg. A dose of 12.5 mg/kg of chlorozotocin produced a 24% reduction, which was not significantly different from control values, but at this dose, two of six treated mice survived > 60 days.

Thus, MCNU was found to cause a much stronger depression of wbc counts than chlorozotocin. However, 12.5 mg/kg of MCNU and 25 mg/kg of chlorozotocin, which were the minimum doses having maximum antileukemic effects, produced a similar depression of wbc counts.

DISCUSSION

Table 7 summarizes the antitumor activities of MCNU. Antitumor activity of MCNU was observed in all experimental tumors such as L1210 leukemia, Lewis lung carcinoma, B16 melanoma, Nakahara-Fukuoka sarcoma, and adenocarcinoma 755. Especially in the early treatment of L1210 leukemia and Lewis lung carcinoma all mice treated with the optimal dose survived > 60 days. Growth-inhibitory and life-prolongation effects of MCNU against established Lewis lung carcinoma were also observed. Lewis lung carcinoma and B16 melanoma are slow-growing but rapidly metastasizing tumors and are rather refractory to chemotherapeutic agents (11,12). In studies on the antitumor activities of nitrosourea compounds against established Lewis lung carcinoma, Mayo et al (13) and Montgomery et al (14,15) have reported that methyl-CCNU exhibited a curative effect and was superior to CCNU or BCNU. Therefore, we examined the antitumor activity of MCNU against Lewis lung carcinoma and B16 melanoma in comparison with methyl-CCNU, since MCNU is a hydrophilic compound and methyl-CCNU is a lipophilic compound and they have different chemical and physical properties. The growth-inhibitory and life-prolongation effects of MCNU and methyl-CCNU against early Lewis lung carcinoma and B16 melanoma were almost the same (table 7). In addition, in established Lewis lung carcinoma, the cure rate using optimal treatment schedules (MCNU: Days 7, 11,

TABLE 6.—Biologic activity of MCNU, chlorozotocin, and CCNU after single ip injection in $BD2F_1$ mice*

Compounds†	Dose (mg/kg)	% lethality in normal $BD2F_1$ mice‡	Nadir wbc count§ (% of control)	L1210 activity (survivors on Day 60/total No. in group)
MCNU	12.5	0	60	6/6
	25	0	34	6/6
	35	0	27	6/6
	50	0	15	3/6
	70	40	8	1/6
Chlorozotocin	12.5	0	76	2/6
	25	0	59	6/6
	50	100	31	0/6
CCNU	50	0	27	6/6

*L1210 system: inoculum = 1×10^5 cells, ip; treatment = Day 1, ip.

†MCNU and chlorozotocin were dissolved in sterile saline. CCNU was suspended in 0.5% carboxymethylcellulose solution.

‡% lethality was based on a 21-day observation period.

§The nadir wbc counts occurred 3 days after injection. Mean wbc counts in controls were 7390 ± 650/mm^3 (saline) and 7460 ± 620/mm^3 (0.5% carboxymethylcellulose).

TABLE 7.—Summary of antitumor activity of MCNU in animal tumors*

	Tumors	Activity of— MCNU	Chloro-zotocin	CCNU	Methyl-CCNU
Survival system	L1210 leukemia	"Cures"†	"Cures"	"Cures"	NT‡
	Lewis lung carcinoma, early	"Cures"	NT	NT	"Cures"
	Lewis lung carcinoma, established	+ +	NT	NT	+ +
	B16 melanoma, early	+	NT	NT	+
	B16 melanoma, established	+ +	NT	NT	+ +
TWI system	Nakahara-Fukuoka sarcoma	+ +	+ +	+ +	NT
	Adenocarcinoma 755	+ + +	+ +	+ + +	NT
	Lewis lung carcinoma, early	+ + +	NT	NT	+ + +
	Lewis lung carcinoma, established	+ + +	NT	NT	+ + +
	B16 melanoma, early	+ + +	NT	NT	+ + +
	B16 melanoma, established	+ + +	NT	NT	+ + +

*In the survival systems an increase in mean survival time (L1210 leukemia) or median survival time (Lewis lung carcinoma, B16 melanoma) over controls was used. In TWI systems an inhibition of tumor weight relative to controls was used. The activity on the optimal schedule for each tumor was graded as follows: – = < 24% ILS or 0–29% TWI; + = 25%–49% ILS or 30%–49% TWI; + + = 50%–99% ILS or 50%–79% TWI; + + + = > 100% ILS or 80%–100% TWI.

†"Cures" = all animals were alive on Day 60.

‡NT = not tested.

and 15, ip; methyl-CCNU: Days 7 and 13, ip) was not much different (30% and 20% respectively). Judging from these results, MCNU seemed to possess almost the same antitumor activity as methyl-CCNU.

Chlorozotocin was reported to be inactive against the early Lewis lung carcinoma but active against the established Lewis lung carcinoma (15). It is of interest that water-soluble MCNU, with a chemical structure similar to that of chlorozotocin, exhibited marked antitumor activity in both early and established Lewis lung carcinoma. In order to determine the therapeutic value of MCNU, the therapeutic ratio was examined in comparison with those of chlorozotocin and CCNU (table 2). With ip administration of MCNU to L1210 leukemia-bearing mice, the therapeutic ratio was apparently higher than that of chlorozotocin or CCNU and almost equal by single or multiple treatment.

It is widely recognized that many nitrosourea derivatives possess myelosuppressive activity which is the main factor limiting their multiple-dose use. Chlorozotocin has already been reported to possess mild bone marrow toxicity (2–5,16–18). In our experiments, we evaluated the bone marrow toxicity of MCNU by determining the peripheral wbc count after a single ip injection in $BD2F_1$ mice compared with chlorozotocin and CCNU. The bone marrow toxicity of MCNU was less than that of CCNU but rather stronger than that of chlorozotocin. However, MCNU possesses a higher therapeutic ratio than chlorozotocin.

The nitrosourea derivative, GANU, in which the *N*-(2-chloroethyl)-*N*-nitrosoureido group is attached to the C-1 position of the glucose moiety (3,19), was effective against L1210 leukemia by oral administration, but chlorozotocin, in which the *N*-(2-chloroethyl)-*N*-nitrosoureido group is attached to the C-2 position, was not (3). Similar results were obtained in our study. We had observed that oral administration of 1-(2-chloroethyl)-3-(methyl-α-D-glucopyranos-2-yl)-1-nitrosourea was effective against L1210 leukemia (7). These four nitrosourea derivatives all have a glucose moiety in their structures, but differ in the position to which the *N*-(2-chloroethyl)-*N*-nitrosoureido group is attached, and also in the presence of a chemical modification of the C-1 OH-group. Substitution of the C-1 OH-group with the OCH_3-group may account for its efficacy by oral administration. Fox et al (18) reported that GANU and chlorozotocin have similar alkylating activities, but the carbamoylating activity of GANU is sevenfold that of chlorozotocin. These results indicate that nitrosourea analogs with a similar chemical structure may differ somewhat in their mode of action, antitumor activity, and toxicity, both quantitatively and qualitatively. From the specific properties and efficacy shown here, MCNU seems to be a good candidate for clinical trials.

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Differential Protection of Normal and Malignant Tissues Against the Cytotoxic Effects of Mechlorethamine [1,2]

John M. Yuhas [3,*]

SUMMARY

The radioprotective drug, WR-2721 (*S*,2-[3-aminopropylamino]ethyl-phosphorothioic acid), has been studied in terms of its ability to (*a*) protect mice against mechlorethamine (HN2)-induced hematopoietic death, and (*b*) alter the ability of HN2 injections to induce growth delay in a solid tumor, the Line 1 lung carcinoma. When WR-2721 was injected ip 15 minutes before iv injections of HN2, it increased resistance to hematopoietic death by a factor of 2, and the protection declined with a half-life of 1.5–2.0 hours. Similar administration of both drugs failed to alter the responsiveness of the Line 1 lung carcinoma to HN2-induced growth delays, except when the HN2 was given within 15 minutes after WR-2721. This interaction of the two drugs, when given within 5–15 minutes of each other, does not appear to be true protection at the tumor site, but rather appears to result from HN2 inactivation in the blood. When HN2 is given 30–60 minutes after WR-2721, it is possible to obtain a twofold increase in the tumor delay without risking increased hematopoietic injury.

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Rapidly acting chemotherapeutic agents, such as mechlorethamine (HN2), rely more on their achievable concentrations than on their duration of action for producing tumor cell killing. Unfortunately, the concentrations which can be achieved clinically, without inducing unacceptable normal tissue complications, are seldom sufficient to eradicate most solid tumors, and the response of the tumor is only partial or temporary. One possible approach to resolving this problem would be to selectively saturate the host's normal tissues with an agent which could inactivate the cytotoxic drug, thereby increasing host tolerance. The merits of this approach are obvious, but, in practical terms, no such selective normal tissue protectors have been available.

The possibility that such drugs did exist was suggested by our own work (1–3) and that of other investigators (4–6) on the development of agents which could selectively protect normal tissues against radiation injury. One drug, WR-2721, was particularly well-suited for application in radiotherapy in that it increased the radiation resistance of murine normal tissues by factors of up to 3, while leaving the resistance of a series of autochthonous and transplanted solid tumors essentially unaltered (7). This selective normal tissue protection would appear to result from the ready absorption of WR-2721 by the normal tissues but deficient drug absorption by the tumor (8,9). In addition, WR-2721 is less efficient as a protector in the presence of hypoxia (10,11), and the critical tumor cells, at least for radiotherapy, may be hypoxic.

Arguing that certain cytotoxic drugs, such as HN2, possess mechanisms of action similar to those of ionizing radiation (12), it appeared reasonable to propose that WR-2721 might protect the normal tissues it concentrates in against HN2-induced injury, while leaving the tumor responses essentially unaltered. We present below our initial analyses on the ability of WR-2721 to protect mice against HN2-induced hematopoietic death and to protect sc transplanted tumors against HN2-induced growth delay.

MATERIALS AND METHODS

The initial studies, which were designed to determine the ability of WR-2721 to protect the mouse against HN2-

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induced hematopoietic death, were conducted at the Biology Division, Oak Ridge National Laboratory, Tenn, in late 1975. These studies employed 4-month-old C57BL/6J female mice which had been purchased from the Jackson Laboratory, Bar Harbor, Me. The toxic LD50 of WR-2721, or the dose which killed 50% of the mice within 10 days, was determined by giving ip injections of graded doses of the drug to five groups of ten mice each. From the resultant mortality data the toxic LD50 was calculated as described below.

For determination of the dose of HN2 which would kill 50% of the injected mice within 30 days, ie, the HN2-LD50(30), at least five groups of eight or more mice each were given graded iv injections of HN2. HN2 was purchased commercially (Mustargen; Merck Sharp and Dohme, West Point, Pa); it was dissolved in 0.9% NaCl immediately prior to use and injected iv. To determine the ability of WR-2721 (sample AN) to protect mice against HN2-induced hematopoietic failure, the drug was dissolved in distilled water immediately prior to use and injected ip (*see* text for doses) between 15 and 120 minutes before the administration of HN2.

In these HN2-LD50(30) assays, deaths were recorded through the 30th day after treatment, but all deaths, with one exception, occurred between the eighth and 17th days after treatment. This mortality pattern is compatible with hematopoietic failure, and we assume below that the HN2-LD50(30) estimates are reflective of hematopoietic sensitivity. The group in which deaths occurred within 72 hours after treatment will be discussed. The 30-day mortality data were used to calculate the HN2-LD50(30) data according to methods described below.

In 1976 we transferred our laboratories to New Mexico and found it necessary to change mouse strains. Before initiating our tumor studies in the new strain, BALB/c, we considered it necessary to confirm our initial estimates of the ability of WR-2721 to protect these mice against HN2. The toxic LD50 of WR-2721 in these mice was determined, and then we determined the ability of WR-2721 to protect against HN2-induced mortality when it was given, under conditions identical to those described above, either 15 or 60 minutes prior to HN2.

Mortality data from the WR-2721 toxic LD50 studies and from the HN2-LD50(30) studies were analyzed according to the method of Finney (13). In order to maintain consistency with our earlier radiation studies (eg, ref 7), the estimates are expressed as the toxic LD50 ± the standard error (SE). The conclusions reached would not be altered if we used the more conventional asymmetric 95% confidence limits. The maximum SE was not > 10% in these studies.

For the analysis of the ability of WR-2721 to inhibit HN2-induced tumor-growth delays, we employed the Line 1 lung carcinoma (14) growing in syngeneic 4-month-old BALB/c female mice. Groups of eight mice each were given an sc transplant of 10^5 cells (15) in the right leg. Eleven days later, when the tumors had reached a mean diameter of 8 mm, treatment was initiated. Treatment consisted of an ip injection of 0.25 ml of saline followed 15 minutes later by graded iv doses of HN2 or ip doses of WR-2721 followed 5–60 minutes later by graded iv doses of HN2. The tumors were sized three times weekly for the next 4 weeks, and the time required for the average tumor to grow from 8 to 12 mm in diameter was calculated. Data are expressed as the delay in time required for this growth (experimental time minus control time) as a function of HN2 dose.

RESULTS

The toxic LD50 for WR-2721 in C57BL/6J mice was found to be 724 ± 17 mg/kg, in agreement with our previously published estimates (1), and drug doses as high as 650 mg/kg failed to induce death. We selected approximately two thirds of the maximum-tolerated dose, or 400

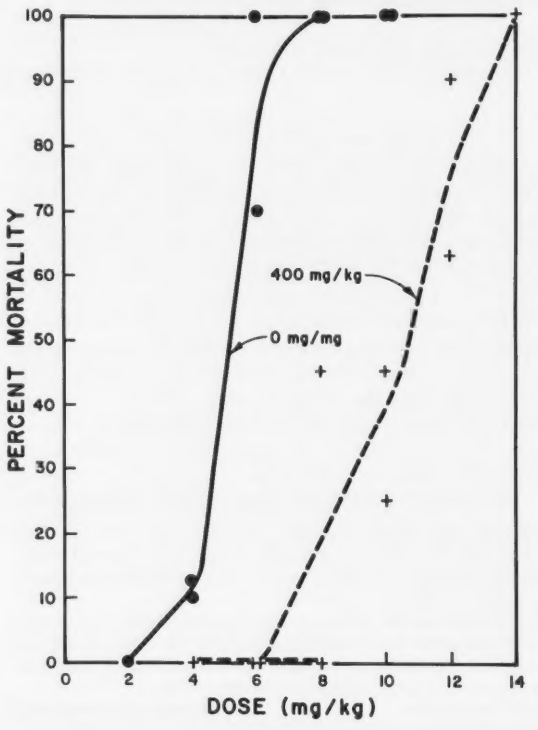

FIGURE 1.—% mortality at 30 days after injection of HN2 with or without ip injection of 400 mg/kg of WR-2721 15 mins earlier.

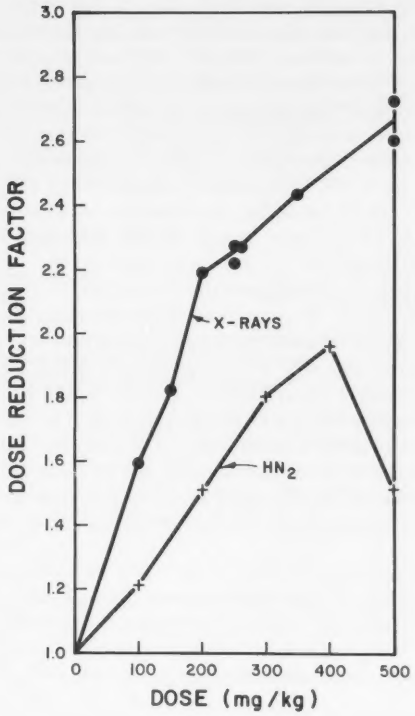

FIGURE 2.—DRF values for 30-day survival in mice given X-rays or HN2 15 mins after injection of graded doses of WR-2721.

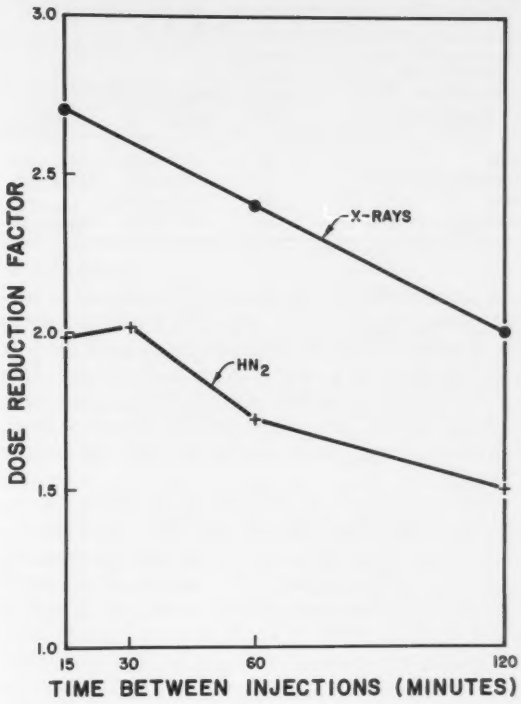

FIGURE 3.—DRF as a function of time following WR-2721 in the X-ray and HN2-LD50(30) systems. Irradiated mice received pretreatment with 500 mg/kg of WR-2721, while HN2-treated mice received 400 mg/kg.

mg/kg, as the dose of WR-2721 to be used in our initial studies on WR-2721 protection against HN2. Figure 1 is a plot of the 30-day mortality observed in C57BL/6J mice as a function of HN2 dose, with or without the ip injection of 400 mg/kg of WR-2721 15 minutes earlier. The HN2-LD50(30) for saline-injected mice was 5.25 ± 0.20 mg/kg, while that for mice that had been pretreated with WR-2721 was 10.90 ± 0.29 mg/kg. Defining the WR-2721 dose-reduction factor (DRF) as the ratio of HN2-LD50(30) values for WR-2721-pretreated and control animals, an estimated DRF of 2.1 (10.90/5.25) is obtained. The SE of the DRF is given by

$$SE = (\{SE_t/LD50(t)\}^2 + \{SE_c/LD50(c)\}^2)^{1/2}$$

(where t and c refer to treated and control groups, respectively) and in this example equals 0.05. Replication of the experiment described above (N = 4) has yielded DRF estimates as low as 1.95 and as high as 2.2.

In figure 2, we have plotted the observed DRF values in the HN2-LD50(30) system as a function of the WR-2721 dose given 15 minutes before HN2 and compared these data to previously published data for WR-2721 in the X-ray LD50(30) system (16). As noted elsewhere (16), protection against X-ray-induced hematopoietic death increases rapidly (0.6% increase in resistance/mg/kg) over the 0–200-mg/kg range, and then more slowly (0.2% increase in resistance/mg/kg) in the 200–500-mg/kg range. Protection against HN2-induced hematopoietic death differs from that observed in the X-ray system in three respects: (*a*) the observed protection is lower at all of the drug doses tested; (*b*) over the range of 0–400 mg/kg, protection increases as a linear function (0.25% increase/mg/kg) of drug dose; and (*c*) overall protection declines between 400 and 500 mg/kg (fig 2; $P < 0.05$). This decline in protection at the 500-mg/kg dose would appear to be an artifact, however, since most of the deaths observed occurred within 72 hours after treatment, suggesting that deaths in this group resulted from a toxic interaction of WR-2721 and HN2, as opposed to HN2-induced hematopoietic failure.

In these initial studies, we also investigated the duration of the ability of WR-2721 to protect against HN2-induced hematopoietic failure. Figure 3 is a plot of the DRFs observed in the X-ray (17) and HN2-LD50(30) sys-

TABLE 1.—HN2-LD50(30) estimates for 4-mo-old BALB/c mice given saline or 350 mg/kg of WR-2721 15 or 60 mins before HN2

Pretreatment	Interval (mins) before HN2	HN2-LD50(30) (mg/kg ± SE)	DRF
Saline	15	3.2 ± 0.17	
350 mg/kg of WR-2721	15	7.0 ± 0.23	2.19 ± 0.06
Saline	60	3.5 ± 0.19	
350 mg/kg of WR-2721	60	6.4 ± 0.23	1.83 ± 0.07

tems as a function of time between the injection of WR-2721 (500 mg/kg for X-rays; 400 mg/kg for HN2) and the time at which X-rays or HN2 was given. Both types of protection decay in a roughly linear manner with a loss of 0.48%/minute in the HN2 system and 0.67%/minute in the X-ray system. These differences in the rate of decay of protection could not be shown to differ significantly ($P < 0.30$).

Upon switching to the BALB/c mouse strain, we redetermined the toxic LD50 for WR-2721, which was estimated to be 754 ± 23 mg/kg. For our studies confirming the ability of WR-2721 to protect against HN2-induced hematopoietic failure, we elected to lower the drug dose to 350 mg/kg in order to avoid the possibility of toxic interaction such as was observed in the high dose range in the C57BL/6J mouse (fig 2). Table 1 summarizes the results of these studies and demonstrates that WR-2721, when given ip 15 or 60 minutes before HN2, offers protection similar to that noted in the C57BL/6J strain under comparable conditions (figs 1 and 2). As with the X-ray-LD50(30) system, the level of protection afforded by WR-2721 is independent of the inherent resistance of the mouse strain studied (16).

The final phase of these initial studies involved the determination of the ability of WR-2721 to protect transplanted tumors against the growth-inhibitory effects of HN2. Mice bearing 8-mm diameter tumors were given either an injection of saline 15 minutes before they received HN2 or 350 mg/kg of WR-2721 5, 15, 30, or 60 minutes before HN2. The choice of the ip route for the WR-2721 and the iv route for the HN2 was made to avoid possible intermixing of the drugs in the circulatory system.

The induced delay in growth as a function of HN2 dose is given in figure 4 for each of the groups. When the mice were given 350 mg/kg of WR-2721 5 or 15 minutes before HN2, the HN2 proved to be less effective in delaying the growth of the tumor than when mice were given saline 15 minutes before HN2. This "protection" of the tumor manifested itself as a shift of the dose-response curve to the right without a significant change in slope. However, when the HN2 was given 30 or 60 minutes after the WR-2721, no tumor "protection" was apparent. Since normal tissue protection persists through 60 minutes after the WR-2721 injection, we clearly have a therapeutic gain, at least with the 30- and 60-minute injection intervals. In order to estimate this gain, we have compared the extent of tumor-growth delay for each group following injection of 50% of the respective HN2-LD50(30) doses. These doses were directly available for control mice (average of two values given in table 1) and for mice given 350 mg/kg of WR-2721 15 or 60 minutes before HN2. The HN2-LD50(30) value for the 5-minute interval was assumed to be the same as that for the 15-minute group, and the 30-minute estimate was obtained by linear interpolation (fig 2) between the 15- and 60-minute values given in table 1. Next, each set of growth-delay data was fit to a linear relationship between HN2 dose and delay, and solved for 50% of the respective HN2-LD50(30) doses. Table 2 shows that the growth delay obtainable following injection of 50% of the respective HN2-LD50(30) doses is greater in mice given WR-2721 15, 30, or 60 minutes before HN2 than in mice given WR-2721 5 minutes before HN2. The gain in tumor response at equal levels of normal tissue injury for the 30- and 60-minute

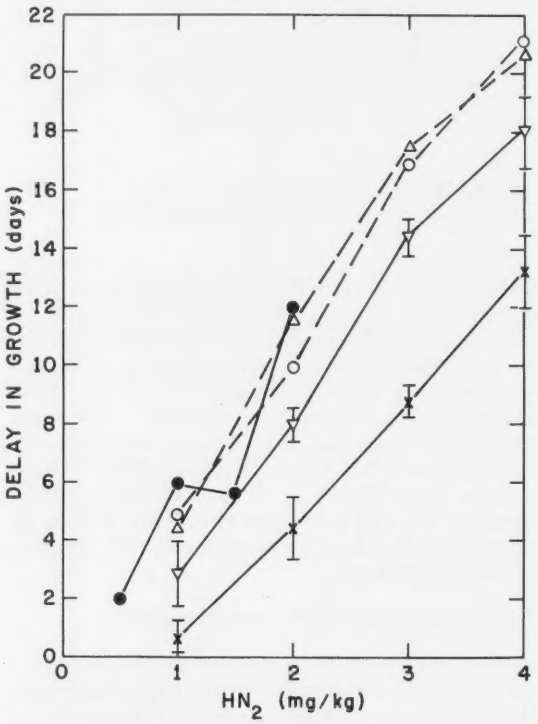

FIGURE 4.—HN2-induced delay in the time required for 8-mm diameter sc transplants of the Line 1 lung carcinoma to grow to 12 mm. (●) = 0.25 ml of saline, 15 mins before HN2. 350 mg/kg of WR-2721 before HN2: (×) = 5 mins before; (▽) = 15 mins before; (△) = 30 mins before; and (○) = 60 mins before.

TABLE 2.—Tumor-growth delay at 50% of the respective HN2-LD50(30) doses in control mice and in mice given 350 mg/kg of WR-2721

Group	HN2-LD50(30) dose (mg/kg)	Growth delay (days) at 50% of HN2-LD50(30)
Controls	3.35	8.9
WR-2721 followed by HN2 in—		
5 mins	7.0*	10.9
15 mins	7.0	16.1
30 mins	6.8†	18.4
60 mins	6.4	17.1

*Assumed to be the same as 15-min estimate.
†Interpolated from 15- and 60-min estimates.

groups represents moderate normal tissue protection in the absence of tumor "protection," while the increase in tumor response in the 15-minute group represents protection of both tissues with the former being favored (table 2).

This ability of WR-2721 to "protect" the tumor when it was given 5 or 15 minutes before HN2 was unexpected, since published data indicate that the drug fails to reach significant levels in the tumor 15 minutes after the injection (8,9). The observed protection could result from rapid tumor absorption and similarly rapid release or from artifactual tumor protection resulting from inactivation of the HN2 in the circulation. However unlikely the former possibility, we wished to obtain at least preliminary direct evidence favoring the latter interpretation. To do so, we injected BALB/c mice with 0 or 350 mg/kg of WR-2721 and harvested their plasma 5–7 or 60 minutes later following decapitation. These plasma samples were diluted 1:1 with 0.9% NaCl containing 1.2 mg/ml of HN2. The final concentration of the HN2 was 0.6 mg/ml, and each test mouse received an HN2 dose of 6 mg/kg. Table 3 summarizes the results of this study which indicate that the plasma of mice given 0 mg/kg of WR-2721 or of mice given 350 mg/kg of WR-2721 60 minutes earlier does not detectably neutralize the HN2 activity, whereas the plasma of mice given 350 mg/kg of WR-2721 5 minutes earlier does. These data are consistent with the argument that the apparent "protection" of solid tumors seen when the HN2 is given 5 minutes after WR-2721 is due, at least in part, to inactivation of the HN2 in the blood.

DISCUSSION

The data presented clearly demonstrate that the injection of WR-2721 before the injection of HN2 can increase the resistance of mice to HN2-induced hematopoietic failure. Further, if the HN2 is not given until ≥ 30 minutes after the WR-2721 injection, no reduction in the ability

TABLE 3.—30-day mortality in mice given 6 mg/kg of HN2 in saline or in 1:1 dilutions of saline and plasma

HN2 diluent	No. of deaths/ No. of mice treated
Saline	8/8
Saline and plasma—	
5 mins after WR-2721*	2/8
60 mins after WR-2721*	8/8
Controls	8/8

*350 mg/kg ip.

of HN2 to inhibit tumor growth is observed. The fact that the tumor-inhibiting effectiveness of HN2 was reduced when it was given within 15 minutes after WR-2721 was totally unexpected. If this observation were the product of true protection at the tumor site, WR-2721 would have to be absorbed by the tumor rapidly and in significant quantities. In addition, we would expect that significant protection of the tumor would be observed when radiation was given within 15 minutes after the WR-2721 injection. All of these expectations are contrary to direct observation, ie, solid tumors do not accumulate significant quantities of WR-2721 (8,9), what little is absorbed is absorbed slowly (8,9), and protection against X-ray injury to the tumor is not observed when the X-rays are given 15 minutes after WR-2721 (*see* ref 7 for review). It would appear more likely that the reduced tumor-inhibiting effectiveness of HN2 when given soon after WR-2721 represents HN2 inactivation in the circulation as opposed to true protection at the tumor site. According to this proposal, significant quantities of the protective metabolite of WR-2721 (this agent must be dephosphorylated in order to protect) persist in the circulation through at least 15 minutes after injection, and these are able to complex and/or inactivate the injected HN2 before it can reach the tumor. Attempts to test this directly, by isolating plasma from mice within 5–7 minutes after WR-2721 injection and determining its ability to inactivate HN2, are consistent with a proposed inactivation in the circulation (table 3).

The experiments presented above were designed to determine whether radioprotective drugs, such as WR-2721, could increase therapeutic effectiveness under idealized conditions. HN2 was chosen as the prototype agent for these studies, since it is rapid in its action and, therefore, closest to X-rays in terms of its duration of action relative to that of WR-2721. Extension of these studies to include agents with a similar mechanism but with a longer duration of action (eg, cyclophosphamide) might not result in as pronounced a therapeutic gain, ie, the protective activity of WR-2721 might decay to minor levels while the concentration of the active form of the cytotoxic drug remains high. Additionally, with regard to the scope of this approach, we have until now consid-

ered chemotherapy as a general topic, but due to the wide range of toxic mechanisms provided by clinically useful cytotoxic drugs, it is not likely that WR-2721 would be protective against all, or even most, types of cytotoxic drug injury. As an example, WR-2721 is totally ineffective in protecting mice against death induced by vincristine, as might be expected from a knowledge of the mechanism of action of the two drugs. Further, hematopoietic tissues are not the limiting ones in all types of chemotherapy, and although WR-2721 offers protection to almost all tissues (except the CNS), it is possible that the level of protection afforded other critical normal tissues will not be sufficient to allow for therapeutic exploitation. Finally, the kinetics of drug absorption and persistence in the circulation could enhance the proposed inactivation of the cytotoxic drug in the circulation, at least for certain agents.

The present experimental results must be viewed therefore with cautious interest, recognizing the aforementioned differences between them and the requirements of the clinic. Nonetheless, it would appear that the potential therapeutic gains which this approach might provide warrant further investigation, especially on the importance or lack thereof of the possible disqualifying factors noted above.

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Phase II Study of VM-26 in Acute Leukemia, Neuroblastoma, and Other Refractory Childhood Malignancies: A Report From the Children's Cancer Study Group [1]

W. Archie Bleyer, William Krivit, Ronald L. Chard, Jr, and Denman Hammond [2]

SUMMARY

VM-26, a semisynthetic podophyllotoxin, was tested for antitumor activity and clinical toxicity in 181 children. The drug was administered iv at weekly intervals, beginning at a dose of 130 mg/m^2/week. The dose was increased, as tolerated, after 3 and 6 weeks to 150 and 180 mg/m^2/week, respectively. The only major toxicity was hematologic, with neutropenia predominating. Anaphylaxis occurred in one patient. The drug demonstrated significant activity in acute lymphocytic leukemia (four responses among 15 patients) and neuroblastoma (ten responses among 31 patients). Objective responses were also noted in one patient each with acute myelogenous leukemia, Hodgkin's disease, histiocytic lymphoma, Wilms' tumor, Ewing's sarcoma, undifferentiated carcinoma, and sacrococcygeal sarcoma. Further trials of VM-26 in these childhood malignancies are warranted.

[Cancer Treat Rep 63:977–981, 1979]

VM-26 is a semisynthetic podophyllotoxin with antineoplastic effects. It is derived from a crystalline extract of the May apple plant, also known as the American mandrake. VM-26 has been shown to be unique among several cell-cycle stage-specific agents tested against resting and rapidly proliferating hematopoietic stem cells to which no absolute resistance could be shown to develop (1). The primary mechanism of cytotoxicity appears to be irreversible blockage of cells in the G_2 phase (2,3), although both metaphase arrest and DNA strand breaks have been reported (4,5).

The Children's Cancer Study Group (CCSG) conducted a phase II trial of VM-26 in children with advanced malignant disease. The purpose of the study was first, to determine which pediatric neoplasms respond to weekly VM-26 (phase II study), and second, to characterize the

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[2]This study was conducted by the Children's Cancer Study Group. The following institutions (with investigators and grant support) participated in the study: University of Southern California Comprehensive Cancer Center, Los Angeles (*D. Hammond, J. Weiner, R. Honour, H. Sather,* and *J. Anderson,* CA-13539); University of Michigan Medical Center, Ann Arbor (*R. Heyn,* CA-02971); Children's Hospital National Medical Center and George Washington University, Washington, DC (*S. Leikin,* CA-03888); Memorial Sloan-Kettering Cancer Center-Cornell University, New York, NY (*D. R. Miller,* CA-14557); Children's Hospital of Los Angeles and University of Southern California (*G. Higgins,* CA-02649); Babies Hospital and Columbia University, New York, NY (*J. Wolff,* CA-03526); Children's Hospital of Pittsburgh and University of Pittsburgh, Pa (*V. Albo,* CA-07439); Children's Hospital of Columbus and Ohio State University (*W. Newton,* CA-03750); Children's Orthopedic Hospital and University of Washington, Seattle (*R. Chard,* CA-10382); University of Wisconsin Hospitals, Madison (*N. Shahidi,* CA-05436); University of Minnesota Sciences Center, Minneapolis (*M. Nesbit,* CA-07306); Children's Memorial Hospital and Northwestern University, Chicago, Ill (*G. Honig,* CA-07431); University of Utah Medical Center, Salt Lake City (*E. Lahey,* CA-10198); Princess Margaret Hospital and University of Toronto, Ontario, Canada (*M. Sonley,* Ontario Cancer Treatment and Research Foundation grant 41–17); Strong Memorial Hospital and University of Rochester, NY (*M. Klemperer,* CA-11174); Children's Hospital and University of Louisville, Ky (*D. R. Kmetz*); University of British Columbia, Vancouver, Canada (*M. Teasdale,* Vancouver Foundation); Children's Hospital of Philadelphia and University of Pennsylvania (*A. Evans,* CA-11796); James Whitcomb Riley Hospital for Children and Indiana University, Indianapolis (*R. Baehner,* CA-13809); New Jersey College of Medicine and Dentistry, Newark (*L. Vitale,* CA-12637); Harbor General Hospital-University of California at Los Angeles, Torrance (*J. Finklestein,* CA-14560); University of California Medical Center, San Francisco (*A. Ablin,* CA-17829); and Rainbow Babies and Children's Hospital and Case Western Reserve University, Cleveland, Ohio (*S. Gross,* CA-20320). All CA grants are from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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toxicity spectrum of an escalating weekly dose regimen (a partial phase I study). The question of cross-resistance with vinca alkaloids was also studied.

PATIENTS AND METHODS

Patients.—The study began in February 1976 and was concluded in February 1978. All patients were under the care of principal investigators of the CCSG located at 22 hospitals in the US and Canada. Patient eligibility was limited to children < 21 years of age with malignant diseases refractory to conventional therapy, including surgery, irradiation, and standard chemotherapy. Only patients with measurable disease were included. Initiation of therapy was delayed in patients with severe granulocytopenia resulting from prior chemotherapy until the absolute neutrophil count rose to > 1000/μl. There was no delay of therapy for patients with neutropenia secondary to malignant disease infiltrating the bone marrow. Thrombocytopenia and anemia did not exclude a patient from entry; platelet and rbc transfusions were administered as needed.

Drug administration.—VM-26 was administered iv over 1 hour at weekly intervals. The starting dose was 130 mg/m^2. If neutropenia occurred as a result of VM-26, subsequent doses were determined by the absolute neutrophil count on the day before the next scheduled dose: no drug was administered if there were < 500 neutrophils/μl; 50 mg/m^2 was administered if there were 500–1000 neutrophils/μl; 100 mg/m^2 was administered if there were 1000–1500 neutrophils/μl; and a full dose was administered if there were > 1500 neutrophils/μl. If the third injection was given at the full dose, the dose was increased to 150 mg/m^2/week for the next 3 weeks. If the latter was tolerated (neutrophil count > 1500/μl), the dose was further escalated and maintained at 180 mg/m^2 weekly.

Criteria for response.—Only responses of ≥ 4 weeks' duration were accepted. In patients with solid tumors, a complete response (CR) was defined as a total disappearance of all recognizable disease. A partial response (PR) was defined as a ≥ 50% decrease in the product of the perpendicular diameters of one or more measurable tumors. Stable disease and subjective response were defined as changes which were less than that described above. Progressive disease was defined as a > 25% increase in measurable lesions or the appearance of new tumors. Hematologic malignancies were quantitated according to the M_1, M_2, and M_3 classification of the leukemias. An M_1 bone marrow was defined as < 5% blast cells in a normocellular marrow with all elements present. An M_3 bone marrow was defined as > 25% blast cells. If the bone marrow improved from M_3 to M_2, the patient was regarded as having a PR, provided the improvement lasted for at least 4 weeks.

Toxicity.—Toxicity was graded on a scale of 1–4, with grade 4 defined as life-threatening. Specific limits for each toxicity grade depended on the organ system and on the patient's age (a copy of these guidelines is available on request from the CCSG). Grade 4 hematologic toxicity was defined as either neutropenia with suspected infection, thrombocytopenia with bleeding or platelet transfusions, or anemia with high-output cardiac failure or rbc transfusion. Grade 3 hematotoxicity was defined as an absolute neutrophil count of < 1000/μl, a total wbc count of < 1500/μl, a platelet count of < 50,000/μl, and/or a hematocrit reading of < 20%, without clinical intervention. Hematologic toxicity was evaluated only in patients without infiltration of the bone marrow. Since most patients were alopecic prior to treatment with VM-26, data on this toxicity were not recorded. Also, phlebitis occurring at the site of injection was not evaluated.

Duration of therapy.—Patients with progressive disease after 3 weeks or with no response after 6 weeks were removed from the study. An adequate trial for both tumor response and clinical toxicity was 21 days.

Required studies.—Weekly studies included physical examination, serum creatinine, BUN, alkaline phosphatase, bilirubin, SGOT, and urinalysis. Hemograms were obtained twice weekly. The staging procedures which documented tumor occurrence prior to VM-26 therapy were repeated at 3-week intervals.

RESULTS

Study Conduct

During the 2 years of the study, 181 patients were entered in the protocol: 59 with acute leukemia and 122 with solid tumors (table 1). That many patients had far-advanced disease was indicated by the 37 patients (20% of those entered) who died of progressive disease before a response could be evaluated, and by the 118 patients (65%) who subsequently died of progressive disease. Additionally, 13 patients who were evaluable for tumor response were so ill that adequate toxicity data could not be obtained. There were few problems with interpretation of the protocol by member institutions, and the overall compliance was excellent. Patient data were unavailable in only three instances. Protocol violations were noted in 12 patients, consisting primarily of patients with no measurable disease, errors in dose or drug intervals, or premature removal of the patient from the study. Twenty-one patients had a CR or PR of at least 4 weeks' duration; responses were noted in 16 patients at the starting dose of 130 mg/m^2 and in five after a dose escalation of 150 mg/m^2.

Efficacy (table 1)

A total of 129 patients (71% of those entered) were evaluable for tumor response, having survived ≥ 21 days

TABLE 1.—Results of weekly VM-26 therapy for childhood cancer

Diagnosis	No. of patients—		No. of patients with—			
	Entered	Evaluable	CR	PR	Stable disease/ subjective response	Progressive disease
Acute lymphocytic leukemia	30	15	1	3	1	10
Acute undifferentiated leukemia	9	5			1	4
Acute myelocytic leukemia, acute myelomonocytic leukemia	19	14	1			13
Central nervous system leukemia	1	1				1
Non-Hodgkin's lymphoma	7	4		1	1	2
Hodgkin's disease	4	4		1	1	2
Brain tumor	17	11			2	9
Wilms' tumor	7	6		1		5
Neuroblastoma	39	31	1	9	6	15
Rhabdomyosarcoma	8	6			1	5
Ewing's sarcoma	8	7		1	3	3
Osteogenic sarcoma	6	6			1	5
Germ cell tumor	3	3			2	1
Retinoblastoma	3	3				3
Carcinoma	6	2		1		1
Sarcoma, miscellaneous	8	7		1		6
Histiocytosis	3	2			1	1
Teratoma	1	0				
Melanoma	1	1				1
Undifferentiated neoplasm	1	1				1
Totals	181	129	3	18	20	88

with adequate tumor measurement and without protocol violation. One half of the patients with acute lymphocytic leukemia (ALL) were not evaluable because of early death (ten patients), protocol violations (three patients), and inadequate recording of clinical data (two patients). Of 15 evaluable patients with ALL, four (27%) had objective responses of at least 4 weeks' duration. There was one CR in a patient who died suddenly, presumably from sepsis, 30 days after remission induction. The dose had been escalated despite severe neutropenia. Consent for autopsy was not granted. Of 14 evaluable patients with acute myelogenous leukemia (AML) or acute myelomonocytic leukemia there was only one response; this patient achieved a CR of 6 weeks' duration after failing to respond to VP-16-213, a closely related podophyllotoxin, as well as vincristine, prednisone, L-asparaginase, 6-mercaptopurine, methotrexate, Adriamycin, cytosine arabinoside, vinblastine, procarbazine, cyclophosphamide, and nitrosoureas.

Ten responses (32%) were observed in 31 evaluable patients with neuroblastoma. One patient with stage IV neuroblastoma had a CR of 31 weeks' duration and nine patients had PRs of 4–28 weeks' duration (median, 7 weeks). Among eight patients with malignant lymphoma, one with Hodgkin's disease had a PR for 7 weeks and one with histiocytic lymphoma had a PR for 4 weeks. Among six evaluable patients with Wilms' tumor, one had a PR of multiple pulmonary metastases. There were no responses in 11 evaluable patients with recurrent brain tumors (medulloblastoma, four; ependymoma, two; astrocytoma, one; brain-stem glioma, two; and brain schwannoma, two). Among the remaining patients, a PR was noted in one patient each with Ewing's sarcoma, undifferentiated carcinoma, and sacrococcygeal small round-cell tumor.

All CRs were documented before the dose of VM-26 was escalated above the starting level of 130 mg/m^2/week. Of the 18 PRs noted, ten were documented only after the dose was escalated to 150 mg/m^2/week and three (neuroblastoma, undifferentiated sarcoma, and Ewing's sarcoma) were documented at the 180-mg/m^2/week dose level.

TABLE 2.—Toxicity of VM-26 in children

Toxicity	No. of patients with toxicity grade—					
	0	1	2	3	4	Totals
Hematologic	17	7	26	14	5	69
Gastrointestinal	101	9	5	1	0	116
Hepatic	100	5	2	0	0	107
Renal	115	1	0	0	0	116
Cardiovascular	114	0	2	0	0	116
Neurologic	115	1	0	0	0	116
Dermatologic	113	2	0	1	0	116
Metabolic	115	0	1	0	0	116
Pancreatic	115	1	0	0	0	116
Fever	114	0	2	0	0	116
Anaphylactic	115	0	0	0	1	116

Cross-resistance with vinca alkaloids was evaluated in patients who failed to respond to vincristine re-induction therapy immediately before entry in the VM-26 study. Five children with acute leukemia had no response to weekly vincristine in combination with prednisone and L-asparaginase during the month before VM-26 therapy. Four of these patients had no response to VM-26 and one had a CR. VM-26 followed vincristine therapy in 17 patients with neuroblastoma (tumor recurrence having developed while they were receiving vincristine every 3 weeks, usually in combination with cyclophosphamide and DTIC); only eight of these 17 patients responded to VM-26. The patients with Ewing's sarcoma, Hodgkin's disease, and Wilms' tumor who had objective responses to VM-26 were previously treated with, and were thought to be resistant to, either vincristine or vinblastine.

Toxicity (table 2)

The major toxicity was hematologic, with grade 2–4 toxicity developing in 45 (65%) of those patients who were hematologically normal before the trial. Myelosuppression was life-threatening in four patients and fatal in one patient. Hematologic toxicity, when encountered, developed primarily after the first dose of VM-26. By the time of the second or third weekly dose, most patients were able to tolerate resumption of a full dose, and subsequent dose escalation, with less toxicity. Neutropenia occurred more frequently than lymphopenia or thrombocytopenia. Anemia was unusual.

Nausea and vomiting were notably infrequent, with six (5%) of those patients who were evaluable having grade 2 toxicity or greater. Vomiting, when it occurred, was generally mild and persisted for < 24 hours. Liver toxicity was rare. Only two patients developed an SGOT elevation of more than twice the normal value. Abnormalities in other liver function tests were not observed. One patient each developed seizures, increased serum amylase, and hypercalcemia. Two patients appeared to have febrile reactions with repeated administration of the drug. A near-fatal anaphylactic reaction developed in one patient within 5 minutes after starting the first infusion of the drug. Elevation of the blood pressure was noted in two patients with no reports of hypotension or other symptoms during the drug infusion. After 90 patients had been treated without any reports of hypotension during the hour of drug administration, the infusion time for each dose was reduced to 30 minutes. The reduction in the infusion duration in 91 subsequent patients did not result in any new toxic effects and did not appear to affect response rates.

DISCUSSION

Weekly VM-26 is active in ALL and neuroblastoma with 27% and 32% response rates (CRs or PRs of ≥ 4 weeks' duration in preterminal patients), respectively. The observation in neuroblastoma is consistent with that reported for the twice weekly dose schedule used at the St. Jude Children's Research Hospital (6). VM-26 appears to have limited activity in acute nonlymphocytic leukemia in that a single response of 6 weeks' duration was noted among 14 evaluable patients. VM-26 appears to be active in some children with AML, Wilms' tumor, histiocytic lymphoma, Hodgkin's disease, and Ewing's sarcoma. The overall significance of the responses in sacrococcygeal nonteratomatous sarcoma and undifferentiated carcinoma is uncertain, in view of the single patients with these diseases who were treated. The putative efficacy of VM-26 in brain tumors (7–9)[3] was not confirmed in a small number of patients. Also, one patient with meningeal leukemia failed to respond. The use of

[3]Holton CP, and Hutter JJ, Jr. Cited in Straus GM, Goldsmith MA, and Slavik M. Clinical brochure: 4'-Demethyl-epipodophyllotoxin-β-D-thenylidene glucoside (NSC-122819), VM-26. Prepared by the National Cancer Institute, Bethesda, Md, Jan 1975.

VM-26 in the treatment of central nervous system neoplasms merits additional evaluation.

Because most of the patients treated in this study who responded were considered to be resistant to either vincristine or vinblastine, VM-26 does not appear to be cross-resistant with the vinca compounds. Additionally, one patient with AML had a CR to VM-26 after completely failing to respond to an adequate trial of VP-16-213, a VM-26 analog. This event, admittedly a single observation, lends credence to the contention that the two drugs are not necessarily cross-resistant (10). Although one comparative analysis of VM-26 and VP-16-213 failed to show any significant differences between the two drugs (11), a preliminary evaluation by the CCSG (12) suggests that this is not entirely the case. In the CCSG analysis, VM-26 appeared to be more active in neuroblastoma and ALL and VP-16-213 was more active in acute myelomonocytic leukemia and rhabdomyosarcoma (12).

Other than myelosuppression, the weekly dose schedule of VM-26 was minimally toxic and relatively easy to administer on an out-patient basis. Children do not appear to be as susceptible to the hypotensive effects of VM-26 reported in the initial adult studies. The drug does not appear to be nephrotoxic or neurotoxic and its hepatotoxicity is minimal. Anaphylaxis, observed in one patient in this study, has been reported previously (13,14). Myelosuppression is by far the major toxic effect, although life-threatening bone marrow depression was largely avoided by escalating the dose as tolerated. Most patients underwent full dose escalation after an initial reduction in drug dose. This pattern suggests induction of a degree of tolerance by normal hematopoietic tissues to VM-26. Overall, the toxicity data suggest that weekly VM-26, without major dose reductions, can be used in combination with other drugs, including moderately myelosuppressive agents.

The wide spectrum of activity in pediatric cancer warrants selected phase III clinical trials in responding tumors. Combinations of VM-26 with other drugs, eg, cytosine arabinoside (15) and nitrosoureas (9), are also indicated. The CCSG has initiated a clinical trial in neuroblastoma of VM-26 combined with *cis*-dichlorodiammineplatinum(II). There may also be a rationale for combined therapy with VM-26 and VP-16-213 because the two drugs have similar antitumor spectrums and yet may not be cross-resistant (10).

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Levamisole in Squamous Cell Carcinoma of the Head and Neck [1]

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SUMMARY

One hundred and thirty-four patients (118 males and 16 females) with epidermoid carcinoma of the head and neck were studied. After treatment of the primary lesion, they were randomized into two groups: 69 received levamisole, 150 mg/day orally for 3 consecutive days every other week, and 65 received placebo. Immune status was also evaluated. Positive reactions to dinitrochlorobenzene increased significantly after primary tumor therapy in both levamisole- and placebo-treated patients. There were no significant differences in immune responses between the two groups, except in recall antigen reactivity, which was decreased in both groups but significantly more levamisole-treated patients became negative ($P < 0.05$). The overall recurrence and death rates at 36 months did not differ between the two groups of patients. However, stage I and II patients treated with levamisole had a significantly higher incidence of recurrence than the placebo-treated patients ($P < 0.02$), while there was some evidence that levamisole-treated stage IV patients did better. It is concluded that the overall outcome in patients with squamous cell carcinoma controlled locally by surgery or radiation was not favorably affected by levamisole in the dose and schedule used in this protocol.

[Cancer Treat Rep 63:983–990, 1979]

The recognition that cancer patients may be immunologically deficient, that immune status may change as a result of therapy, and that immunologic status is of prognostic value are all of importance to the design of clinical cancer therapy trials. Many trials currently being performed attempt to increase the host immune response as well as decrease the effects of immunosuppressive therapy (1–4).

Epidermoid carcinomas of the head and neck are associated with a high incidence of defects in cell-mediated immunity (5–8) and there is evidence that these defects are related to unfavorable therapeutic results. For example, Eilber et al (5) have suggested that disease recurrence is more frequent in patients who remain dinitrochlorobenzene (DNCB)-negative than in those who were originally DNCB-positive. It has also been shown that radiotherapy impairs the immune response and that few irradiated (9) or surgically treated (10,11) patients attain responses comparable to control patients, even though their disease remains controlled (12).

Such results suggest that agents which enhance the immune response may have a role in the clinical management of head and neck cancer. Levamisole (1,10,12),[4] which was introduced in 1966 by Thiepont as an anthelmintic, can affect immune modulation both in vitro (13,14) and in vivo (15,16). Encouraged by favorable results obtained with levamisole in irradiated stage III breast carcinoma (17), we have conducted a randomized, double-blind, placebo-controlled, prospective clinical study for patients with epidermoid carcinoma of the head and neck after primary local control of the disease by radiation or surgery.

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[4]Amery WK. Levamisole action as an immunotherapeutic agent. Janssen Pharmaceutica publication No. 12235.

MATERIALS AND METHODS

Patient Population

A total of 143 patients with epidermoid carcinoma of the head and neck were entered in the study between August 1974 and August 1977. Nine were excluded from subsequent analysis either because they were lost to followup or because of protocol violations. The remaining 134 patients (118 males and 16 females) were analyzed.

Staging and Treatment

All patients were staged by the TNM classification of the International Union Against Cancer (UICC) (1974). For primary locations of tumors for which the UICC does not recommend staging, the American Joint Committee staging was used. Treatment of the primary lesion was carried out using combinations of radiotherapy and surgery as appropriate to the site and stage. Patients free of gross disease after radical treatment were randomized to receive either levamisole (150 mg/day orally for 3 consecutive days every other week) or placebo on the same schedule; treatment was stopped when recurrence occurred. Clinical followup examination was carried out at monthly intervals during the first year and every 2 months thereafter. Laboratory tests and roentgenograms were performed as needed to assess the disease.

Immunologic Studies

Skin reactivity to DNCB (25 and 100 μg), recall antigen reactivity (*Candida* 1/1000 and streptokinase/streptodornase 40/10), and lymphocyte in vitro blastic transformation with phytohemagglutinin were carried out at 3-month intervals. The technique and interpretation of these studies have been previously described (7).

Statistics

The statistical analysis was performed by evaluating the difference between percentages when $n_1 \cdot p_1$, $n_2 \cdot p_2$, $n_1(100 - p_1)$, and $n_2(100 - p_2)$ were all > 5; if not, the appropriate chi-square test was used. However, if in one inner cell there was a value of < 5, the Fisher exact probability test was used. Actuarial curves were compared using the Wilcoxon test. $P \leq 0.05$ was considered to be statistically significant.

RESULTS

A total of 69 patients were randomized to receive levamisole and 65 to receive placebo. The primary locations of the tumor for patients in the two groups are shown in table 1. Oral cavity, oropharynx, and larynx were the most frequent sites. Skin primaries were present in significantly greater numbers in the placebo group ($P < 0.05$). The age distribution of the patients in the two groups is given in table 2. They did not differ significantly and the majority of patients were > 50 years of age (78.5% in the placebo group and 76.8% in the levamisole group). There was no difference in the amount of alcohol or tobacco consumption between the two groups. Stage of disease was also comparable in the levamisole and placebo groups (table 3) with similar numbers for early (46.2% for placebo and 44.9% for levamisole patients) and advanced (53.8% for placebo and 55.1% for levamisole patients) stages. Also, the types of primary treatment received by the two groups were equivalent (table 4).

Skin Reactions

Following primary treatment of the tumor, there was an increase in positive reactions to 100 μg of DNCB in both the placebo and levamisole groups (table 5). Weak and negative reactions diminished in frequency and strong reactivity increased markedly ($P < 0.001$). There were no differences between the levamisole and placebo groups, however, with respect to skin reactivity.

There were no recurrences among patients in the placebo group, whose reactions to 100 μg of DNCB became positive after 6 months. By contrast, a 36.4% recurrence rate was found in the group treated with levamisole (table 6). This difference nearly attained statistical significance ($P = 0.085$).

Recall Antigens

After primary treatment, skin reactivity to recall antigens showed a general decrease over the time span of the study. This was more marked in the levamisole-treated patients. Twenty levamisole-treated patients developed negative reactions compared to eight in the control group ($P < 0.05$) (table 7). Since a similar number of patients were treated by radiotherapy alone or combined with surgery in both groups, this decrease was analyzed in both, and it was found that five of 51 placebo patients (9.8%) showed this decrease compared to 18 of 62 levamisole-treated patients (29%) ($P < 0.05$).

There was also a prognostic significance attached to this decrease. Patients who maintained a strong reactivity during the entire period of the study had a recurrence rate of 5.6% (one of 18 patients), whereas in the group of patients who had decreased reactivity or never had a strong reaction the rate of recurrence was 31.9% (37 of 116 patients) ($P = 0.012$).

Blastic Transformation

There was a difference in the blastic transformation observed prior to treatment between the placebo and

TABLE 1.—Primary location of disease

Site	No. of placebo-treated patients (%)	No. of levamisole-treated patients (%)	*P* value
Skin	9 (13.9)	2 (2.9)	0.05
Oral cavity	24 (36.9)	28 (40.6)	NS
Oropharynx	10 (15.4)	13 (18.8)	NS
Nasopharynx	1 (1.5)	0	NS
Hypopharynx	2 (3.1)	2 (2.9)	NS
Paranasal sinuses	0	2 (2.9)	NS
Larynx	19 (29.2)	19 (27.5)	NS
Unknown primary	0	3 (4.3)	NS

TABLE 2.—Age of patients

Age (yrs)	No. of placebo-treated patients (%)	No. of levamisole-treated patients (%)	*P* value
< 40	3 (4.6)	2 (2.9)	NS
40–50	11 (16.9)	14 (20.3)	NS
51–60	15 (23.1)	23 (33.3)	NS
61–70	18 (27.7)	18 (26.1)	NS
> 70	18 (27.7)	12 (17.4)	NS
Total	65 (100.0)	69 (100.0)	

TABLE 3.—Disease stages

Treatment	No. of patients (%) with—			
	Stage I	Stage II	Stage III	Stage IV
Placebo	14 (21.5)	16 (24.6)	27 (41.5)	8 (12.3)
Levamisole	16 (23.2)	15 (21.7)	34 (49.3)	4 (5.8)

TABLE 4.—Primary treatment

Treatment	No. of placebo-treated patients (%)	No. of levamisole-treated patients (%)	*P* value
Radiotherapy	31 (47.7)	30 (44.9)	NS
Radiotherapy + surgery	20 (30.8)	32 (46.4)	NS
Surgery	14 (21.5)	7 (10.1)	NS
Total	65 (100.0)	69 (100.0)	

levamisole groups (table 8). There was a higher pretreatment incidence of poor blastic transformation in the group randomized to receive levamisole than in the group receiving placebo (43.9% versus 25.6%, $P < 0.05$). After treatment there was a slight increase in good blastic transformation in the levamisole group at the expense of the fair responses (19.3% compared to 27.3%), while the poor responses remained unchanged. In the placebo group there was an increase in poor blastic transformation after treatment and a corresponding decrease in fair transformation. These variations in blastic transformation do not have statistical significance either within groups or between groups.

Within the placebo group there was no correlation between improved blastic transformation and the incidence of recurrence: 15.7% among patients with good blastic

TABLE 5.—Variation in responses to DNCB (100 μg) after primary treatment

Treatment	Before treatment: No. of patients (%)	After treatment: No. of patients (%)	*P* value
	Negative reactions		
Placebo	15 (23.1)	8 (12.3)	NS
Levamisole	24 (34.8)	10 (14.5)	0.05
P value	NS	NS	
	Weak reactions		
Placebo	32 (49.2)	12 (18.5)	0.05
Levamisole	27 (39.1)	11 (15.9)	0.05
P value	NS	NS	
	Strong reactions		
Placebo	18 (27.7)	45 (69.2)	0.001
Levamisole	18 (26.1)	48 (69.6)	0.001
P value	NS	NS	

TABLE 6.—Recurrences as related to time of positive reaction to DNCB (100 μg)

Positive reaction—	Placebo-treated patients		Levamisole-treated patients		*P* value
	NED*	Recurrence (%)	NED*	Recurrence (%)	
Before 6 mos	17	4 (19.0)	21	5 (19.2)	NS
After 6 mos	8	0	7	4 (36.4)	0.085
Always strong	12	4 (25.0)	11	3 (21.4)	NS

*NED = no evidence of disease.

TABLE 7.—Variation in reactivity to recall antigens after treatment

Treatment	No. of patients with increased reactivity (%)	No. of patients with decreased reactivity (%)
Placebo	0	8 (12.3)
Levamisole	3 (4.3)	20 (29.0)
P value	NS	0.05

TABLE 8.—Variation in blastic transformation after treatment

Treatment	Before treatment: No. of patients (%)	After treatment: No. of patients (%)	*P* value
	Poor		
Placebo	11 (25.6)	21 (41.2)	NS
Levamisole	25 (43.9)	22 (40.0)	NS
P value	0.05	NS	
	Fair		
Placebo	16 (37.2)	12 (23.5)	NS
Levamisole	21 (36.8)	18 (32.7)	NS
P value	NS	NS	
	Good		
Placebo	16 (37.2)	18 (35.3)	NS
Levamisole	11 (19.3)	15 (27.3)	NS
P value	0.05	NS	

transformation or who improved their blastic transformation versus 9.1% among patients who had a decreased response. These results were reversed in levamisole-treated patients, with recurrences occurring in 35.3% of patients who responded poorly or decreased their responses compared to 7.7% recurrences among patients whose blastic transformation improved ($P = 0.08$).

Survival and Recurrence

At 36 months there were 17 recurrences (24.6%) and four deaths (5.8%) in levamisole-treated patients and 11 recurrences (16.9%) and six deaths (9.2%) in the placebo patients. These differences were not statistically significant. Actuarial curves for the disease-free interval (fig 1) showed that 69.8% of placebo patients and 67.3% of levamisole-treated patients were free of disease at 36 months (not significant).

Since there were more skin cases in the placebo group ($P < 0.05$), rates of recurrences and death were also calculated with these patients excluded. The results were similar, with a recurrence rate of 28.6% for placebo patients and 31.3% for levamisole patients (not significant). Actuarial curves for these two groups showed that 67.3% of the placebo and 66.1% of the levamisole patients were free of disease at 30 months (not significant).

When recurrence rates were correlated with age, the levamisole group showed more recurrences in the 40–50-year age group than in the > 70-year age group, but these differences were not statistically significant ($P = 0.079$ and $P = 0.12$).

The relationship between recurrence and stage of disease is shown in table 9. Levamisole-treated patients had a higher recurrence rate than placebo-treated patients for stages I and II combined ($P < 0.02$), while both groups had a similar recurrence rate in stage III and the levamisole group did better in stage IV (M_0), although not at a level of statistical significance ($P = 0.14$). The actuarial curves for disease-free interval for stages I and II showed that at 30 months no placebo patients had had recurrence, while only 75.8% of levamisole patients were disease-free ($P < 0.02$) (fig 2). Although the recurrence rates for both groups were similar for stage III, the actuarial curves for disease-free interval showed 37.5% relapses in the levamisole-treated group compared to 24.5% in the placebo group (not significant) (fig 3).

Toxicity

There was mild toxicity in seven patients treated with levamisole (10.1%). Symptoms consisted of nausea, increased or abnormal olfactory perception, headache, and general malaise. These symptoms were not persistent and cleared spontaneously in all cases. More severe toxicity was found in three patients, in whom these symptoms plus vomiting were severe enough to force discontinuation of levamisole within the first 3 months of treatment. No hematologic toxicity was detected in any patient.

DISCUSSION

This study evaluated the immunologic and clinical status of patients with head and neck cancer. The observed increase in DNCB skin reactivity that occurred in both levamisole and placebo patients could reflect an improvement in immune competence as a result of primary control of their tumors. Of particular interest was the observation that there were no recurrences among patients in the placebo group whose skin reactivity to 100 μg of DNCB became positive 6 months after treatment, whereas there was a 36.4% recurrence rate among similar patients treated with levamisole. A possible explanation of this difference is that the increased skin reactivity in the levamisole-treated patients is due not only to improved immune competence, but also to other actions of levamisole that will need further elucidation.

At the same time, antigen skin reactivity showed a decrease that was more marked in the levamisole group. Decline in skin reactivity to recall antigens was associated with a prognostically important high rate of tumor recurrence. In addition, the analysis of data regarding blastic transformation indicated that there was no correlation between tumor recurrence and improved blastic transformation in the placebo-treated group, but there was a definite correlation between tumor recurrence and poor blastic transformation in the levamisole group.

TABLE 9.—Recurrences related to disease stage

Stage	Placebo-treated patients		Levamisole-treated patients		*P* value
	NED*	Recurrence (%)	NED*	Recurrence (%)	
I	14	0	14	2 (12.5)	0.02
II	16	0	10	5 (33.3)	
III	16	11 (40.7)	21	13 (38.2)	NS
IV	2	6 (75.0)	3	1 (25.0)	0.14

*NED = no evidence of disease.

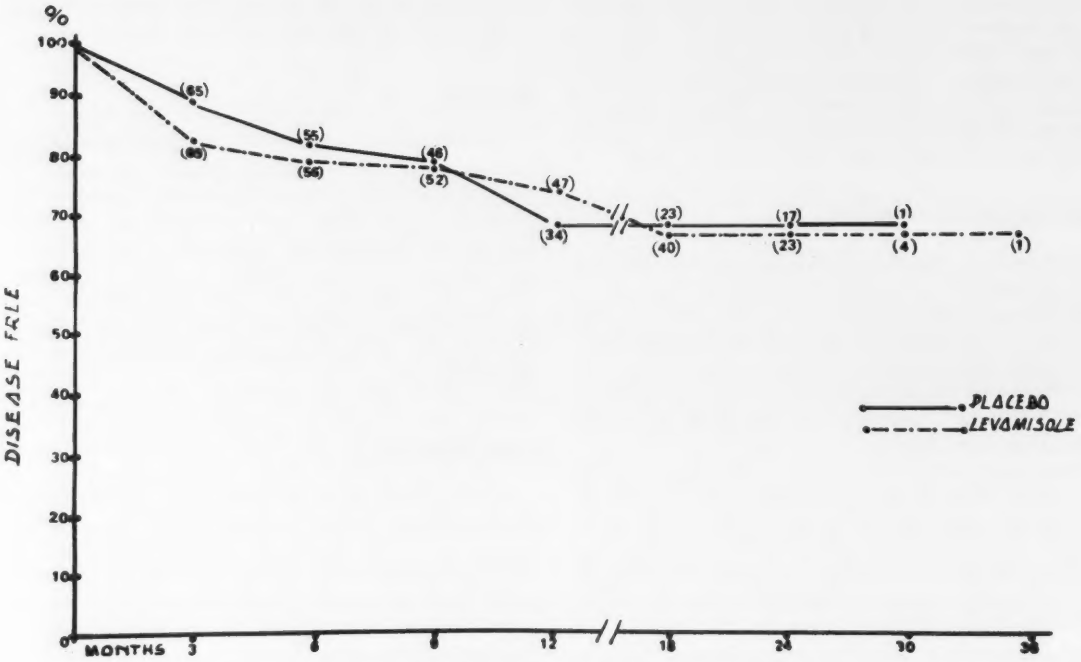

FIGURE 1.—Actuarial curves for disease-free interval for all patients.

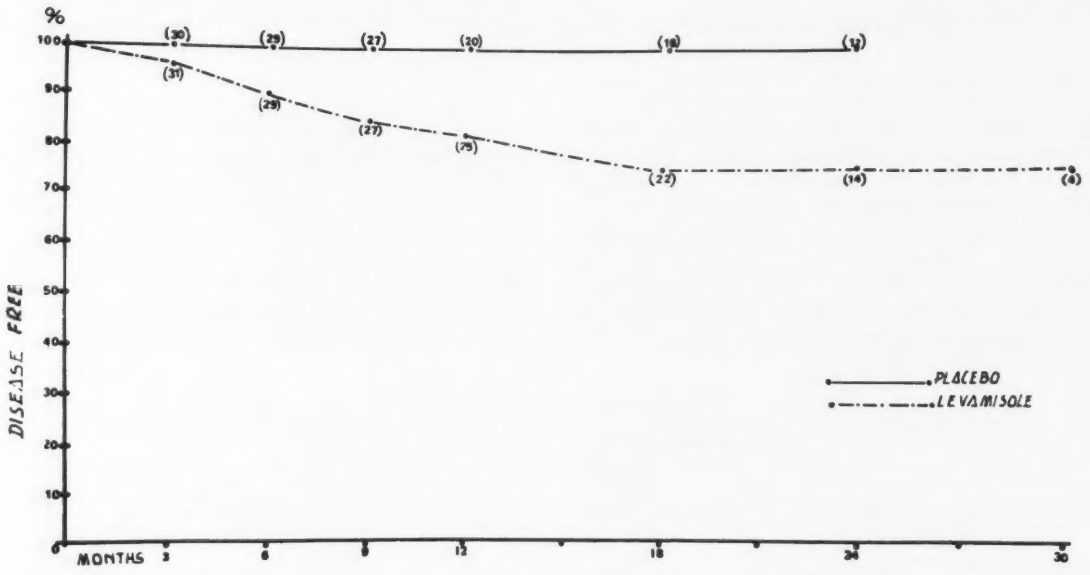

FIGURE 2.—Actuarial curves for disease-free interval for stages I and II.

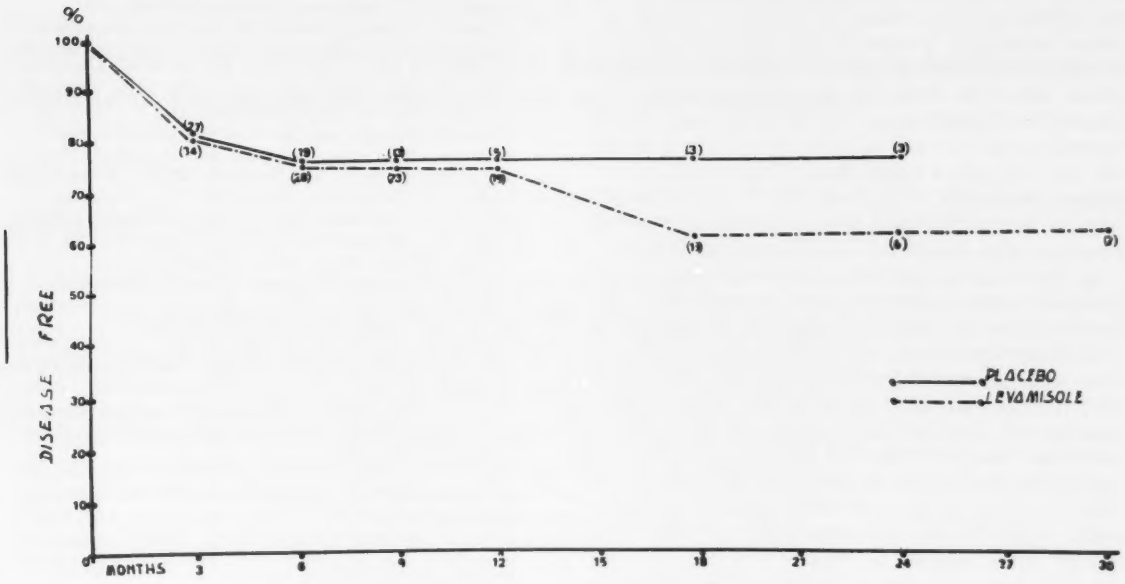

FIGURE 3.—Actuarial curves for disease-free interval for stage III.

These findings can be partially explained by specific immunologic features of squamous cell carcinoma of the head and neck. For example, there is not a linear relationship between decreases in T-cell numbers and increases in tumor bulk (18). Also, while quantitative increases in T lymphocytes have been described after successful radical treatment (19), individual variations that range from values above those of the control population to minimal or no recovery are common. It is also known that smokers, who represent a large majority of this series, have increased numbers of T lymphocytes as well as better responses to phytohemagglutinin than nonsmokers (20). All of these considerations become more involved when levamisole patients are analyzed, since this drug has been found to have widely variable actions on different aspects of immune competence. These include no influence on blastic transformation of human lymphocytes (21), increased protein synthesis in human peripheral lymphocytes (22), increased human macrophage phagocytosis and lymphocyte proliferation (23), and decreased phagocytosis of particles of colloidal ^{198}Au in rats (24). It is concluded, therefore, that further work is needed to determine the real meaning of the variations in blastic transformation found in these groups.

Analysis of recurrence and survival in both groups as a whole shows a remarkable similarity, indicating that levamisole in this dose and schedule does not alter the course of disease recurrence and survival. Disease-free actuarial curves for the entire group show 69.8% of placebo patients and 67.3% of levamisole patients free of disease at 30 months, but differences appear when the actuarial curves are analyzed by stage. As stated earlier, for stages I and II at 24 months there is a 24.5% difference in recurrence rate ($P < 0.02$), and for stage III, although the number of recurrences is similar at the end of the study, at 24 months the levamisole group has 13% more recurrences, which seems to indicate an earlier relapse in treated patients. There was a nearly significant increase ($P = 0.08$ and $P = 0.12$) in incidence of recurrence in the 40–50-year group and the > 70-year group treated with levamisole.

The significant increase in recurrence rates in stages I and II in levamisole-treated patients may imply a drug-induced change in host-tumor relationships which impaired the chance of the host controlling subclinical disease. By contrast, stage IV patients, although small in number, have shown a lower rate of recurrence than placebo patients. Although with our present knowledge it is difficult to interpret these findings, these results should preclude the use of levamisole in early-stage disease, while suggesting that it may be of use in advanced cases after radical treatment. This is similar to our previously published experience in breast cancer (14).

The dose of levamisole used was the same for all patients: 150 mg/day on 3 consecutive days every other week. Others have proposed 25 mg/kg or 100 mg/m^2 (*see*

footnote 4) in the same schedule. It is improbable that this difference would reduce the immunomodulatory action of levamisole, because it was demonstrated to be effective in human breast cancer (17) as well as in breast cancer induced in female Sprague-Dawley rats by 7,12-dimethylbenz(*a*)anthracene. In the latter case, tumor regression was observed with low doses, 2 and 4 mg/kg/day, but not with a higher dose, 8 mg/kg (25). Nevertheless, the results of this study and the lack of knowledge on the mechanism of action of levamisole warrant a revision of its assessment as an immune modulator.

In 1976 this group published experience with stage III irradiated breast cancer (17) which demonstrated a favorable action of levamisole in significantly lengthening both disease-free interval and survival. Re-evaluation of these same patients in December 1976 (26) and October 1977 (unpublished data) has confirmed these results. Undoubtedly, head and neck squamous cell carcinoma and breast cancer are quite different biologic entities and such differences explain the absence of favorable results with levamisole in head and neck squamous cell carcinoma. In this respect, our experience is similar to the smaller series reported by Wilkins et al (27), in which five of six levamisole-treated patients had recurrence. It differs, however, from the results found in another series[5] where survival in levamisole-treated patients at 40 months was 60% compared to 20.5% in control patients. However, those authors did not state the composition of the patient groups or the modalities of radical treatment used.

In conclusion, this study demonstrates that head and neck squamous cell carcinoma patients with disease controlled by surgery or radiation are not favorably affected by levamisole in this dose and schedule. However, it may be worthwhile to continue studying the effects of levamisole in stage IV patients.

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Levodopa and Dopamine Analogs: Melanin Precursors as Antitumor Agents in Experimental Human and Murine Leukemia [1,2,3]

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SUMMARY

L-Dopa methyl ester has been shown to be a novel antitumor agent. Furthermore, the L-dopa analogs, D-dopa, α-methyldopa, and dopamine, also exhibit significant antitumor activity in the L1210 and P388 lymphocytic leukemia systems. Structure-activity studies confirmed that the presence of a catechol moiety was essential for activity. Two analogs, 3,4-dihydroxybenzylamine and *N*-acetyldopamine, which were much less neurotoxic, exhibited the greatest antitumor activity. In vitro, at concentrations from 0.5 to 3.0 mM, there is a rapid and profound inhibition of radiolabeled thymidine incorporation as compared to uridine or leucine incorporation. Continuous exposure of exponentially growing L1210 cells to L-dopa methyl ester at similar concentrations for up to 24 hours resulted in a block of traverse of cells through the cell cycle in G_1 coupled with a depletion of cells with a G_2 complement of DNA. In vivo, toxicity of these compounds appears to be mediated principally by conversion to dopamine. Similar effects upon thymidine incorporation were observed in human leukemia cells in vitro.

[Cancer Treat Rep 63:991–997, 1979]

L-Dopa has been shown to be selectively toxic to human pigmented melanoma cells in vitro (1). Conversion of L-dopa to its ester has resulted in significant in vivo antitumor activity in the following experimental tumor systems: (*a*) L1210 lymphocytic leukemia, (*b*) P388 lymphocytic leukemia, (*c*) B16 melanoma, and (*d*) C1300 neuroblastoma (2,3). It was postulated that the mechanism of action involved oxidation to a quinone with subsequent sulfhydryl group scavenging and inhibition of enzymes central to DNA synthesis. This hypothesis was supported by the well-known ability of L-dopa to form covalent adducts with cysteine and the profound and selective inhibition of thymidine incorporation observed in our studies. Much of the in vivo toxicity of L-dopa could be attenuated by pretreatment of mice with inhibitors of the enzyme aromatic L-amino acid decarboxylase. This enzyme mediates a principal catabolic route of L-dopa, ie, the conversion of L-dopa to dopamine (4).

In order to extend our previous observations and to define the structural parameters necessary for the activity of these novel antitumor agents, we have examined the effect of analogs upon the growth of L1210 and P388 leukemias. The first group included D-dopa, α-methyldopa, and dopamine, agents which have marked neurologic activities. The second group included 3,4-dihydroxybenzylamine and *N*-acetyldopamine, where structural modifications have eliminated the neurologic activity (5).

α-Methyldopa and dopamine were of interest since they are currently used extensively in man for the treatment of vasomotor disorders, a factor which would make their clinical application particularly facile. 3,4-Dihydroxybenzylamine and *N*-acetyldopamine were chosen in an attempt to reduce the neurotoxicity observed in earlier studies while retaining the antitumor effect.

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MATERIALS AND METHODS

Drugs.—All drugs used in this study were reagent grade and were obtained from Sigma Chemical Co, St. Louis, Mo. Benserazide (Ro4–4602) was a gift of the Hoffmann-LaRoche Co, Nutley, NJ. Radiolabeled (methyl-^{3}H)-thymidine (specific activity, 40–60 curies/mmol), (5-^{3}H)-uridine (specific activity, 40–60 curies/mmol), and L-(4,5-^{3}H)-leucine (specific activity, 20 curies/mmol) were from the New England Nuclear Corp, Boston, Mass. All other materials were reagent grade and were used without further purification.

In vivo antitumor assays.—BDF_1 ($C57BL/6 \times DBA_2)F_1$ male mice, 6–8 weeks of age, were obtained from the Jackson Laboratory, Bar Harbor, Me. All drugs were administered ip in 0.9% sodium chloride except for *N*-acetyldopamine which was prepared in alcohol, Emulphor, and 0.9% sodium chloride (1:1:8). Antitumor activity was determined by measuring the increase in lifespan (ILS) in mice given either 1×10^5 L1210 or 1×10^6 P388 lymphocytic leukemia cells ip as compared to sham-injected controls. Assay procedures were identical to those of standard National Cancer Institute protocols (6). In all experiments, the treatment was given daily for 7 days, beginning on the day after tumor inoculation. The percent ILS was calculated according to the formula $\%ILS = (T/C - 1) \times 100$, where T is the median survival time in days of the treatment group and C is the median survival time in days of the control group. Significance tests were performed by analysis of variance.

In vitro assays.—L1210, P388, and human CCRF-CEM lymphocytic leukemia cells were a gift from Dr. H. Lazarus (Sidney Farber Cancer Institute) and their maintenance has been described in detail (7). Briefly, the cells were maintained in suspension in Eagle's minimum essential medium containing 15% fetal bovine serum, 100 μg/ml of streptomycin, and 100 units/ml of penicillin in a 5% CO_2 humidified air incubator at 37°C. The medium was also supplemented with 0.05 mM 2-mercaptoethanol. The techniques for radiolabeled precursor incorporation studies have also been described (7). A total of 5×10^5 cells/ml were exposed to 1 microcurie/ml of labeled precursor and the appropriate concentration of drug for 60 minutes. The cells were then filtered onto a nitrocellulose filter (Millipore Corp, Bedford, Mass) and washed once with 10% trichloroacetic acid and three times with 0.9% sodium chloride. The filters were dried overnight at 60°C, placed in scintillation vials containing Aquasol (New England Nuclear Corp), and counted in a Beckman LS335 scintillation counter. All determinations were in triplicate with values expressed as the mean ± SEM for three to five experiments and compared to nontreated controls.

Cell cycle progression.—The effect of L-dopa methyl ester upon cell cycle traverse was analyzed by means of sequential DNA histograms determined by flow cytofluorometric analysis. This method, utilizing propidium iodide for DNA staining, has been described (8). Exponentially growing cells were treated with various concentrations of drug, harvested after 6, 18, and 24 hours by centrifugation at $800 \times g$ for 10 minutes, and resuspended in propidium iodide solution. A total of 400,000 cells was measured for each determination. The results are expressed as a histogram representing the relative number of cells with a given DNA content.

RESULTS

Table 1 depicts the results of a single-dose toxicity study on normal BDF_1 mice. As previously observed, L-dopa methyl ester at doses > 1000 mg/kg is quite toxic. In general, immediately following injection the animals became hyperexcitable and tremulous, and displayed signs consistent with a hypercatecholamine state. Deaths from drugs were erratic among experiments and with repeated injections tolerance could be induced. Pretreatment of the animals with benserazide, a potent inhibitor of the enzyme aromatic L-amino acid decarboxylase, completely abolished these signs and permitted the delivery of much higher doses, eg, 2000 mg/kg, without measurable toxicity.

The use of the analogs D-dopa methyl ester and α-methyldopa methyl ester, both agents that are not readily decarboxylated by the enzyme, permitted the delivery of much higher doses. Similarly, the analog 3,4-dihydroxybenzylamine, a derivative lacking sympathetic agonist activity, was also much less toxic. Finally, dopamine administered preformed was less toxic than L-dopa methyl ester.

In Vivo Antitumor Studies

Table 2 summarizes the results of in vivo antitumor activity studies using the L1210 and P388 systems. L-

TABLE 1.—Comparison of in vivo toxicity and in vitro inhibition of L1210 cells by L-dopa analogs

Compound	LD50* (mg/kg)	IC50 (L1210)† (mM)
L-Dopa methyl ester	1000 (4.05)‡	0.30
Dopamine	1250 (6.61)	0.19
α-Methyldopa methyl ester	1750 (6.36)	0.36
D-Dopa methyl ester	> 2400 (8.10)	0.35
3,4-Dihydroxybenzylamine	> 2400 (9.09)	0.31
N-Acetyldopamine	> 2400 (10.25)	0.15

*Represents the results of a single-dose toxicity study on normal BDF_1 mice (30/group) observed for 30 days.

†Concentration that caused a 50% inhibition of growth of treated L1210 cells as compared to nontreated controls following 48 hrs of continuous exposure.

‡Values in parentheses = mmols/kg.

TABLE 2.—Antitumor activity of L-dopa analogs against L1210 and P388 leukemias

Drug	Dose* (mg/kg)	L1210 Range of survival (days)	L1210 %ILS†	P388 Range of survival (days)	P388 %ILS†
L-Dopa methyl ester	500	3–13	33	15–20	46
	600	4–14	33	14–20	27
D-Dopa methyl ester	1000	10–12	22	13–16	36
	1500	10–15	33	14–16	45
α-Methyldopa methyl ester	750	10–12	22	4–18	11
	1000	10–13	33	4–20	20
Dopamine	400	11–15	44	13–17	41
	600	3–13	22	14–21	50
3,4-Dihydroxybenzylamine	600	11–15	53	15–23	60
	800	12–17	65	16–24	70
N-Acetyldopamine	600	12–15	52	15–24	64
	800	12–18	66	16–25	80

*Mice were inoculated with either 10^5 L1210 cells or 10^6 P388 cells ip and were treated ip daily, Days 1–7.

†Calculated according to the formula (T/C – 1) × 100, where T is the median survival time in days of treated group and C is the median survival time in days of control group. Acceptable control survival = 8.5–9 days (median) (range, 8–11 days). Toxic deaths were defined as occurring in treated groups before the control median for the schedules above; this was never > 10% of the animals in each test group.

Dopa methyl ester demonstrates significant activity ($P < 0.01$) at a dose of 500 mg/kg. α-Methyldopa methyl ester, although less toxic, exhibits significant activity ($P < 0.01$) although less than that of L-dopa methyl ester. D-Dopa methyl ester, at a daily dose of 1000 mg/kg, exhibits both less toxicity and a minimally enhanced antitumor response in the P388 system. Dopamine itself is observed to have significant antitumor activity in both L1210 and P388 leukemias. Neither tyrosine methyl ester nor 3-methoxytyrosine exhibited antitumor activity.

The most active compounds are in fact *N*-acetyldopamine and 3,4-dihydroxybenzylamine. It is apparent that the increased activity is at least partially due to decreased toxicity as evidenced by the range of survival of treated animals.

Table 3 represents the results of a preliminary study of the effect of schedule upon response of L1210 leukemia to the analog 3,4-dihydroxybenzylamine. It is apparent that more intensive treatment for shorter periods which is made possible by decreased toxicity results in improved therapeutic response. Dopamine, when administered in a similar schedule at 200 and 400 mg/kg, resulted in > 50% toxic deaths. A similar relationship is also observed in the case of the DNA polymerase inhibitor cytosine arabinoside.

In Vitro Antitumor Assays

Table 1 depicts the effects of each analog upon the growth of L1210 cells following 48 hours of continuous exposure. The results expressed as IC50 (50% inhibitory concentration) reveal minimal differences among the agents. It is apparent from table 1 that the most effective agents (3,4-dihydroxybenzylamine and *N*-acetyldopamine) combine effective in vitro inhibitory activity with markedly reduced in vivo toxicity.

Figure 1 depicts the temporal course of thymidine incorporation by L1210 cells in vitro. During the 60-minute exposure, untreated cells incorporate thymidine linearly. Simultaneous exposure to L-dopa methyl ester at a concentration of 0.5 mM results in a marked reduction in the rate of incorporation to approximately 50% of control. Higher concentrations of 1.0 and 3.0 mM cause a rapid inhibition of thymidine incorporation, beginning as soon as 5 minutes after exposure. There is also a corresponding inhibition of cell growth paralleling thymidine inhibition.

Table 4 summarizes the results of our studies on the effects of L-dopa analogs upon the incorporation of ra-

TABLE 3.—Effect of schedule on response of L1210 leukemia to 3,4-dihydroxybenzylamine

Dose (mg/kg)	Schedule*	%ILS†
200	q3h × 8	40 (10)
400	q3h × 12	50 (25)
200	q3h × 3 (Days 1–4)	66 (0)
400	q3h × 3 (Days 1–4)	66 (20)
600	q3h × 3 (Days 1–4)	88 (30)

*Treatment was initiated on Day 1 following implantation of 10^5 cells (10 mice/group, ip injection).

†Nos. in parentheses = % 60-day survivors. There were no toxic deaths as defined by animals dying before controls. %ILS is based upon dying animals only.

TABLE 4.—Effect of L-dopa analogs on thymidine, uridine, and leucine incorporation by L1210 cells in vitro

Drug	% inhibition								
	Thymidine*			Uridine*			Leucine*		
	0.5 mM	1.5 mM	3.0 mM	0.5 mM	1.5 mM	3.0 mM	0.5 mM	1.5 mM	3.0 mM
Tyrosine methyl ester		0			0			0	
L-Dopa	14 ± 9	62 ± 8	72 ± 6	0	0	2 ± 1	8 ± 4	10 ± 6	10 ± 4
α-Methyldopa	9 ± 3	36 ± 5	49 ± 2	0	2 ± 5	5 ± 11	3 ± 3	14 ± 6	15 ± 4
D-Dopa	5 ± 3	23 ± 8	66 ± 3	0	2 ± 3	5 ± 4	5 ± 1	6 ± 3	14 ± 6
L-Dopa methyl ester	34 ± 3	94 ± 1	98 ± 1	4 ± 3	11 ± 8	22 ± 4	6 ± 2	13 ± 2	17 ± 1
D-Dopa methyl ester	40 ± 8	88 ± 3	90 ± 3	7 ± 10	14 ± 6	13 ± 3	5 ± 5	8 ± 4	7 ± 3
α-Methyldopa methyl ester	16 ± 7	59 ± 6	77 ± 5	0	6 ± 6	17 ± 11	5 ± 1	13 ± 4	30 ± 9
3-Methoxytyrosine		0			0			0	
Dopamine	16 ± 7	79 ± 6	94 ± 1	2 ± 2	9 ± 9	10 ± 3	2 ± 2	15 ± 5	18 ± 9
3,4-Dihydroxybenzylamine	90 ± 5	98 ± 1	98 ± 3	5 ± 4	10 ± 4	15 ± 5	0	0	
N-Acetyldopamine	81 ± 5	95 ± 2	98 ± 2	26 ±	28 ± 5	39 ± 10	11	16	26

*Values represent mean cpm ± SE for 3 separate determinations and represent an incubation of 60 mins for each precursor. cpm/10^4 cells for controls were: thymidine, 640 ± 31; uridine, 842 ± 40; and leucine, 50 ± 2.

diolabeled thymidine, uridine, and leucine. There is a profound and rapid inhibition of thymidine incorporation observed with L-dopa methyl ester, with little effect upon uridine or leucine incorporation. Neither tyrosine methyl ester nor 3-methoxytyrosine demonstrated any inhibitory activity.

When compared on a molar basis, D-dopa methyl ester is approximately as potent as L-dopa methyl ester as an inhibitor of thymidine incorporation while α-methyldopa methyl ester is somewhat less effective. In each instance, however, it is apparent that there is a selective inhibition of thymidine incorporation as compared to uridine or leucine incorporation. Furthermore, there is greater inhibition observed with the ester as compared to the corresponding free acid while dopamine, 3,4-dihydroxybenzylamine, and *N*-acetyldopamine are the most potent inhibitors.

Table 5 presents the results of comparison of effects of L-dopa analogs on L1210 and human leukemic cells. Although not shown, the pattern of inhibition of uridine and leucine was also similar for each line. The major inhibition occurred with thymidine incorporation while leucine and uridine incorporation were much less affected at each concentration. These data, in addition to previous data upon human melanoma cells, suggest that the inhibitory phenomenon is shared by human and murine cells.

Cell Cycle Progression Studies

Figure 2 displays the DNA histograms of cells treated with the parent compound, L-dopa methyl ester. At 6 hours, there is a dose-related decrease in the peak corresponding to cells with a G_2 content of DNA, and at 18 hours there is an increase in the number of cells in G_1 suggesting augmentation of this population by cells that were in G_2 and continued their traverse through mitosis unaffected by drug and subsequently arrested in G_1. Computer-assisted analysis of curves at 6 hours revealed a G_1 population of 52%, 54%, 58%, and 60% and a G_2 + M population of 20%, 12%, 8%, and 6% for control at 0.5, 1.5, and 3.0 mM drug concentrations respectively. Similar results were obtained with the human leukemic cells. These observations, in addition to thymidine inhibition studies, lend strong support to the concept of DNA synthesis inhibition as a primary site of action. At 1.5 and 3.0 mM, there was no increase in the total number of cells, indicating that proliferation is completely turned

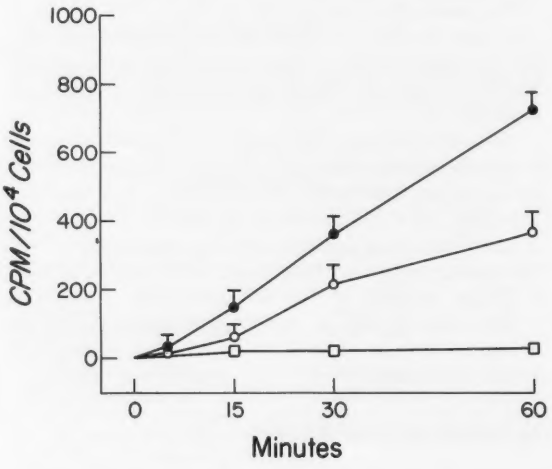

FIGURE 1.—Effect of L-dopa methyl ester on thymidine incorporation by L1210 cells in vitro. ● = control, ○ = 0.5 mM, □ = 1.0 mM.

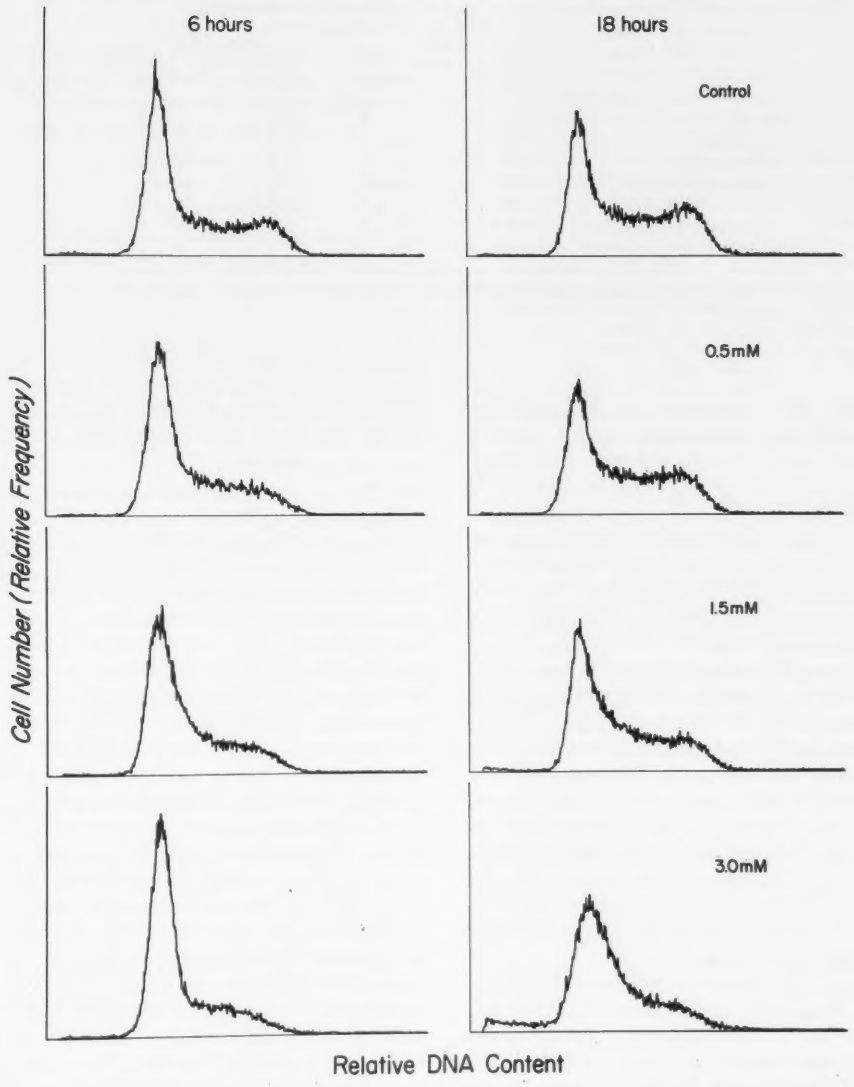

FIGURE 2.—DNA histograms of L1210 cells following 6 and 18 hrs of exposure to L-dopa methyl ester. At 6 hrs there is a decrease in the peak composed of cells with a G_2 complement of DNA. At 18 hrs, there is augmentation of G_1 at each concentration.

off. Furthermore, this effect remains constant as long as drug exposure is maintained, and at 24 hours viability as measured by trypan blue exclusion fell to 0.

At 0.5 mM, however, there is some leakage of cells through the block and hence, a G_2 peak develops again. Consistent with this observation is the slow (about 15% of control) proliferation of these cells, indicating impaired but present ability to proliferate. At 24 hours, these cells also are < 10% viable as indicated by dye exclusion. These results confirm the thymidine incorporation studies where drug at 1.5 and 3.0 mM completely inhibits proliferation while the 0.5-mM dose is only partially effective.

TABLE 5.—Comparison of effects of L-dopa analogs on thymidine incorporation by human leukemic and murine leukemic cells

	Thymidine incorporation* (% inhibition)			
	L1210		CCRF-CEM	
	0.5 mM	1.0 mM	0.5 mM	1.0 mM
L-Dopa	15 ± 8	58 ± 8	12 ± 5	56 ± 4
Dopamine	17 ± 6	80 ± 4	21 ± 6	76 ± 8
α-Methyldopa	10 ± 2	30 ± 4	15 ± 4	25 ± 4
L-Dopa methyl ester	32 ± 4	92 ± 5	32 ± 8	90 ± 5
D-Dopa methyl ester	40 ± 7	86 ± 4	44 ± 8	87 ± 2
α-Methyldopa methyl ester	18 ± 7	60 ± 4	15 ± 4	56 ± 5
3,4-Dihydroxybenzylamine	91 ± 2	96 ± 1	90 ± 5	98 ± 1

*Control cells incorporated 620 ± 5 cpm/10^4 cells (L1210) and 420 ± 15 cpm/10^4 cells (CCRF-CEM).

DISCUSSION

L-Dopa methyl ester represents the first-described member of a novel class of antitumor agents. Furthermore, as expected, D-dopa methyl ester and α-methyldopa methyl ester are significantly less toxic, presumably as a result of their resistance to decarboxylation. There is not, however, an appreciable increase in antitumor activity observed since the intrinsic ability to inhibit thymidine incorporation is also somewhat less. Dopamine, when administered preformed, is less toxic than L-dopa methyl ester, suggesting that the toxicity observed in the case of L-dopa methyl ester is only partially mediated through conversion to dopamine. Part of the explanation for this phenomenon may be the relative impermeability of the blood-brain barrier to dopamine as compared to L-dopa methyl ester, thereby permitting entry of catecholamine into brain with L-dopa methyl ester but not with dopamine. Substitution on the α-carbon atom, although having large effects upon in vivo pharmacology, has relatively little effect upon in vitro activity. Esterification of the amino acid in each substance markedly enhanced activity, which is likely due to the increased solubility and pKa of the drugs.

The results with the analogs 3,4-dihydroxybenzylamine and *N*-acetyldopamine suggest that it might be possible to separate the neuroagonist effect of these agents from their antitumor effects and that a substantial increase in activity is achieved. In each instance, the catechol portion was retained, suggesting that the inhibitory effects are caused by oxidation while neuroagonist effects were eliminated by removing a methylene group or acetylating the nitrogen. These are structural modifications that are known to abolish neural activity (5).

In our studies, we have confirmed that D-dopa, α-methyldopa, and dopamine share antitumor activity in experimental tumor systems and also the property of inhibition of thymidine incorporation, suggesting that an enzyme central to DNA synthesis might be the site of action. In addition to the selective inhibition of thymidine incorporation, the observed rapid onset of G_1 block of the traverse of cell cycle also supports the idea of inhibition of DNA synthesis as a primary site of action. It is apparent from these observations that cells that have already completed their DNA synthesis (G_2) were able to undergo division and reaccumulate in G_1 where they were subsequently blocked.

The concept that a DNA polymerase itself is the target of these compounds is further supported by recent work of Graham et al (9) and Vogel et al (10). They showed that mammalian DNA polymerase is highly sensitive to the inhibitory effects of preformed 1,2-benzoquinones, and, furthermore, that this inhibition could be reversed by exposure to potent sulfhydryl-reducing agents. In their study, the quinone derived from *N*-acetyldopamine was also more potent than that derived from L-dopa as an inhibitor, a finding which correlates with antitumor activity. Our results are completely consistent with these observations and suggest, furthermore, that it is not essential to administer a quinone; a quinol itself has antitumor activity, possibly through intracellular conversion to the quinone. Presumably, L-dopa analogs are oxidized to the corresponding orthoquinone, a reaction that might be facilitated by the presence within the cell of a unique biochemical attribute, eg, tyrosinase activity within melanoma cells. The quinone then reacts with a nucleophile, such as a free sulfhydryl group, thereby irreversibly inactivating a specific enzyme. It is apparent from our study that leukemia cells themselves are capable of being inhibited by catechols. The mechanism by which leukemic cells themselves might oxidize the catechols is unclear although peroxidases are present in many types of cells (3).

It is of course possible, and in fact likely, that these agents act at more than one site, with the enzymes succinic dehydrogenase, α-ketoglutaric dehydrogenase, and pyruvic dehydrogenase also exhibiting sensitivity to sulfhydryl reagents (11). Alternatively, these agents might act through the autoxidation of catechol to quinone, thereby producing superoxide and hydroxide free radi-

cals (12). These highly reactive species in turn would be cytotoxic by interaction with DNA and cell membranes.

Additional observations in the literature may also be explained by our results. It has been observed that the culture medium of pigmented melanoma cells contains a substance that inhibits the proliferation and thymidine incorporation of both melanoma cells and fibroblasts (13). It is possible that at least part of this activity is due to levodopa secreted into the medium.

In summary, the development of this novel class of antitumor agents is interesting for several reasons. First, at least three of the earliest identified members of this class, L-dopa, α-methyldopa, and dopamine, have been used extensively in man and therefore may have an early clinical evaluation. Second, since conversion to the quinone appears to be a necessary activation step, it might be hoped that these agents would be particularly active against those tumors that possess a special biochemical mechanism for oxidizing catechols. This prediction has already been substantiated by the observed activity against melanoma and neuroblastoma (14,15). Finally, the phenomenon described here may have broader implications with respect to the other therapeutic uses of L-dopa, ie, in the treatment of Parkinson's disease, and the role of dopamine in the central nervous system.

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Combination of Adriamycin and Cyclophosphamide in the Treatment of Metastatic Prostatic Carcinoma: A Phase II Study [1,2]

Brief Reports and Preliminary Communications

Ronald M. Izbicki, Magid H. Amer, and Muhyi Al-Sarraf [3,*]

SUMMARY

A prospective trial of Adriamycin and cyclophosphamide was undertaken in the treatment of patients with hormonally refractory prostatic carcinoma. Of the 20 patients evaluable, three (15%) had a partial remission and five (25%) had stable disease for a total response of 40%. All patients who responded had subjective improvement consisting of weight gain (≥ 10% of body weight), improvement of performance status (≥ 10% in Karnofsky index), and a decreased need for pain medication.

[Cancer Treat Rep 63:999–1001, 1979]

Endocrine-resistant prostatic carcinoma is a common clinical entity. Chemotherapy trials in this disease have been frustrating because of difficulty in defining objective criteria by which response could be evaluated. Both Adriamycin and cyclophosphamide (1,2) have been reported effective in the treatment of patients with prostatic carcinoma. This combination has also had a limited trial in prostatic carcinoma (3). A prospective study of both agents was done.

MATERIALS AND METHODS

Twenty-one patients with histologically confirmed adenocarcinoma of the prostate and distant metastasis were entered in this study. One patient was unevaluable because of early death due to pulmonary embolism during the first week of treatment.

The ages of the patients ranged from 53 to 74 years with a median of 68 years. All patients had a life expectancy of 12 weeks. Patients with symptomatic heart disease were excluded.

Previous hormonal therapy consisted of orchiectomy alone in five, both orchiectomy and stilbestrol in 16, corticosteroids in five, and progesterone in two. Eight patients had prior radiotherapy to the pelvis of ≥ 3000 rads.

Pretreatment evaluation consisted of a history and physical examination, blood chemistry studies (including serum acid and alkaline phosphatase, serum creatinine, and blood urea nitrogen), chest roentgenogram and skeletal survey, bone scan, electrocardiogram, and excretory urogram. A cbc and a platelet count were done weekly for the first course and then every 3 weeks. Blood chemistries were repeated at 3-week intervals as was the clinical examination. Skeletal survey and chest roentgenogram were repeated at 12-week intervals.

All prescriptions for pain medication were written on a pad with carbon copies put on the clinic chart. All pain prescriptions written prior to and during chemotherapy were evaluated.

The dose of medication given was 40 mg/m^2 of Adriamycin and 500 mg/m^2 of cyclophosphamide by iv push for good-risk patients and 30 and 400 mg/m^2 of Adriamycin and cyclophosphamide respectively for poor-risk patients. Good-risk patients were defined as those < 65 years of age, not having received ≥ 3000 rads of pelvic radiation, and with no other signs of poor bone marrow reserve. When a total dose of 450 mg/m^2 of Adriamycin was reached, the patients continued to receive cyclophosphamide alone.

A complete remission was defined as the disappearance of all clinically detectable soft tissue tumor, a recalcification of all osteolytic metastases, and a return of an elevated acid phosphatase to normal. A partial remission consisted of > 50% reduction in all measurable soft tissue disease, a return of an elevated acid phosphatase to normal, and 50% recalcification of lytic bone disease. Patients who had a reduction of measurable disease of 25%–50% and no deterioration in weight or performance status were considered to be objectively stable.

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RESULTS

Toxicity

Leukopenia (< 4.0 × 10^3 wbcs/mm^3) was seen in 17 of 20 patients (85%) and thrombocytopenia (< 100 × 10^3 platelets/mm^3) was seen in nine of 20 patients (45%). The median wbc count nadir was 3.1 × 10^3 cells/mm^3 (range, 1.1–5.4) and the median platelet count nadir was 92 × 10^3 cells/mm^3 (range, 51–410). Recovery from hematologic toxicity was seen in 21 days in 13 patients and in 28 days in the remainder. Alopecia was universal. Nausea and/or vomiting was reported in 18 of 20 patients (90%). Two patients refused further therapy because of this side effect. Two episodes of congestive heart failure occurred and resulted in one death. The total doses of Adriamycin in these two patients were 120 and 160 mg/m^2 respectively. Both patients had abnormal electrocardiograms prior to therapy: one had ST segment changes and the other had complete left bundle branch block.

Clinical Response

There were no complete responses; partial objective responses were seen in three of 20 evaluable patients (15%) (patients 6, 7, and 8). A decrease in the size of prostatic nodules occurred in two patients and a decrease in the size of inguinal lymph nodes occurred in one. Objectively stable disease during therapy was seen in five patients (25%) (patients 3, 11, 12, 18, and 19). Subjective response consisting of an improvement in the Karnofsky index of ≥ 10% accompanied by weight gain of ≥ 10% of the initial body weight and/or a decreased need for pain medication occurred in all patients who had a partial response or stable disease (40%). In six of the eight patients reporting subjective improvement, pain relief occurred 3–5 days after the treatment and exacerbated shortly before the time of the next dose of medication (on or about Days 17–21). Some of the patient characteristics before and during chemotherapy are shown in table 1.

The acid phosphatase level was elevated in 14 patients including six responders. In the six patients who responded, the acid phosphatase level normalized in one, decreased by ≥ 50% in two, rose in one, and did not change in the remaining two. In those patients with progressive disease the acid phosphatase level dropped in three and increased in the remainder. No patient with depression of bone marrow elements due to tumor had normalization while receiving treatment.

The median duration of response was 19 weeks with a range of 5–40 weeks. The median survival was 40 weeks for patients responding to therapy versus 17 weeks for nonresponders ($P = 0.05$).

TABLE 1.—Patient characteristics before and during chemotherapy

	Status before therapy					Status at maximal effect of therapy				
Patient No.	Performance status	Wt (lbs)	Acid phosphatase (IU/liter)	Alkaline phosphatase (IU/liter)	Measurable disease*	Response† (objective/subjective)	Performance status	Wt (lbs)	Acid phosphatase (IU/liter)	Alkaline phosphatase (IU/liter)
1	80%	132	1.52	63	Lungs, liver	I/I	50%	124	1.32	—
2	70%	150	0.32	38	Bone	I/I	60%	140	0.37	26
3	70%	172	5.1	111	Bone, liver, nodes, local	N/P	80%	169	7.9	1090
4	60%	113	48.5	314	Bone, local	I/I	60%	112	30.0	107
5	60%	93	22.36	490	Bone, local	N/N	60%	92	28.94	—
6	60%	174	6.02	222	Bone, local	P/P	80%	182	0.40	—
7	60%	144	17.6	192	Bone, local	P/P	70%	155	3.2	90
8	80%	218	0.92	41	Nodes, local	P/P	90%	227	4.2	—
9	70%	162	3.2	274	Bone, local	N/I	50%	160	5.5	150
10	70%	200	1.34	130	Bone, local	N/N	60%	198	2.9	80
11	50%	118	0.8	52	Bone, local	N/P	70%	127	1.6	48
12	60%	154	4.04	170	Bone, local	N/P	70%	165	13.7	120
13	60%	171	0.68	280	Bone, local	N/N	60%	176	—	—
14	50%	89	0.81	110	Bone, local	N/I	50%	88	—	—
15	80%	168	24.02	53	Bone, local	I/I	60%	165	12.5	—
16	70%	180	7.38	110	Bone	I/I	70%	180	9.40	100
17	80%	128	0.8	—	Bone, local	N/N	70%	125	0.8	55
18	60%	152	39.9	110	Bone, local	N/P	70%	167	10.49	66
19	60%	150	58.8	70	Bone, local	N/P	80%	165	56.7	—
20	70%	148	4.2	—	Bone, local	Not evaluable	—	—	—	—
21	60%	161	12.05	68	Bone, local	I/I	60%	155	6.8	65

*Local = prostatic nodule or enlargement.
†P = partial response; N = no change; I = increasing disease.

DISCUSSION

Patients with hormonally refractory disseminated prostatic cancer are usually included in systemic chemotherapy trials. Evaluation of the efficacy of such trials is sometimes difficult because of the lack of measurable disease.

Recently, the National Prostatic Cancer Project issued criteria for evaluation which include changes in performance status, weight, and the use of pain medication as part of the evaluation for response (4).

The adaptation of these criteria had stimulated chemotherapeutic trials with a variety of agents. Benefit in metastatic prostatic cancer has been observed in 36%–83% of patients treated with a variety of chemotherapeutic regimens (5–10).

In our study utilizing Adriamycin and cyclophosphamide, a 15% partial remission rate was seen in patients with measurable disease and an additional 25% of patients subjectively improved and gained weight (objective stable disease). The hematologic toxicity from this combination was tolerable in our group of patients, many of whom had had pelvic radiation and/or myelopthisis.

The serum acid phosphatase level did not correlate with the clinical response in our patients. This has also been observed by others (9). The utilization of an immunologic method for measuring acid phosphatase may clarify the usefulness of this enzyme as a marker of response (11,12).

It was concluded that the combination of Adriamycin and cyclophosphamide is effective in patients with advanced prostatic cancer after failure to respond to hormonal manipulation. Further study is needed to compare this combination with other active agents.

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Amelioration of Adriamycin Skin Necrosis: An Experimental Study[1]

Brief Reports and Preliminary Communications

Martin H. Cohen[2,3,*]

SUMMARY

Skin necrosis following subcutaneous Adriamycin extravasation is a significant clinical problem. In this study Adriamycin dilution and various drugs were tested for their ability to ameliorate this toxic effect. When Adriamycin was diluted to a concentration of ≤ 0.25 mg/ml, no skin ulceration was observed. Drugs such as hydrocortisone, dexamethasone, lidocaine, bupivacaine, phentolamine, and hyaluronidase were ineffective in modifying Adriamycin skin necrosis. Care in iv drug administration and dilution of Adriamycin are the best means of preventing the sequelae of subcutaneous Adriamycin extravasation.

[Cancer Treat Rep 63:1003–1004, 1979]

Extravasation into subcutaneous tissues from Adriamycin infusions may result in severe local necrosis (1). Empirically, drugs such as hydrocortisone or local ice applications have been used to ameliorate this toxic effect (2). There is no evidence, however, that such treatment is beneficial. The present study was designed to determine the efficacy of various drugs in decreasing the local toxicity of sc administered Adriamycin.

MATERIALS AND METHODS

Male CDF_1 mice, supplied by the Charles River Breeding Laboratory (Boston, Mass), were used. Mice were 6–8 weeks old and weighed between 23 and 28 g. Adriamycin was administered sc through a 25- or 26-gauge disposable needle in a volume of 0.5 ml. The injection site started at the popliteal fossa of the right leg with infiltration to the right femur. Skin changes following sc Adriamycin administration were measured as the products of the perpendicular diameters of hair loss over the injection site as well as the product of the perpendicular diameters of skin ulceration.

Adriamycin

Adriamycin was diluted with preservative-free normal saline. In an initial dose-response study Adriamycin solutions containing 1.0, 0.5, 0.25, or 0.125 mg/ml were administered in a volume of 0.5 ml. For subsequent attempts to modify Adriamycin skin toxicity the Adriamycin concentration was 0.5 mg/ml. Two or three mice were used for each drug dose and schedule tested.

Drugs used to ameliorate Adriamycin skin toxicity (table 1) were administered into the Adriamycin infiltration site in a volume of 0.5 ml. The initial drug treatment was always given within 3 minutes of the time of Adriamycin infiltration. Controls for each drug administration consisted of animals receiving Adriamycin alone. Additional control mice received the therapeutic drugs alone in an identical manner to the mice receiving Adriamycin and therapeutic drug.

RESULTS

Dose-response studies revealed that Adriamycin concentrations of 0.25 and 0.125 mg/ml were not associated with hair loss or skin ulcerations. At Adriamycin concentrations of 0.5 and 1.0 mg/ml skin toxicity was initially manifested by local inflammation noted within several hours of drug administration. Local hair loss and early skin ulceration were not seen until 5–7 days after Adriamycin administration. Skin ulceration progressed over the next 1–2 weeks and then stabilized. Skin ulcers healed in all animals after ≥ 5 weeks. The mean sizes of skin ulcers in mice receiving 1 and 0.5 mg/ml of Adriamycin were 1.2 and 0.72 cm^2, respectively. The area of hair loss was two to three times as large as the area of skin ulceration.

None of the drug treatments listed in table 1 prevented the development or decreased the size of skin ulcers. At the higher doses of hydrocortisone, skin ulceration was delayed for 1–2 days. The time to healing of ulcers was also not affected by any of the treatment programs. Skin

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TABLE 1.—Dose and schedule of drugs used to ameliorate Adriamycin skin toxicity

Drug	Dose (mg/ml)	Schedule*
Hydrocortisone	100†	A
Sodium succinate	50†	A
Solu-Cortef	25	A
	10	B, C
Dexamethasone	2	B, C
Lidocaine	2 or 4	B, C
Bupivacaine	1 or 2	D
Phentolamine	1	D, E
Hyaluronidase	10	D

*A = single dose; B = daily × 1, 2, or 4 days; C = twice daily × 1, 2, or 4 days; D = daily × 1, 2, or 3 days; and E = twice daily × 1, 2, or 3 days.

†Toxic dose: mice died within 24 hrs of injection.

ulcerations were sometimes larger in mice receiving twice daily drug administration but this probably resulted from repeated needle punctures. None of the drug treatments produced ulcers in control mice not receiving Adriamycin.

DISCUSSION

None of the drug treatments used in this study significantly modified Adriamycin-induced skin necrosis. Hydrocortisone and dexamethasone were chosen for study because of their known anti-inflammatory and membrane-stabilizing effects (3) and because they have been frequently used empirically. The local anesthetics, lidocaine and the longer-acting bupivacaine, also have membrane-stabilizing effects (4). Phentolamine, an α-adrenergic blocking agent, prevents epinephrine-associated skin necrosis (5) and hyaluronidase has been shown to limit ischemic myocardial injury by enhancing transport of metabolic substrates to the ischemic area (6).

When Adriamycin was diluted to a concentration of ≤ 0.25 mg/ml, no skin ulceration occurred. This observation may have clinical applicability, although more dilute solutions would necessitate a longer infusion time than is usually used. Whether prolonged infusion would significantly change Adriamycin pharmacokinetics would also have to be determined.

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Chemotherapy for Advanced Cervical Cancer With Methotrexate, Bleomycin, and *cis*-Dichlorodiammineplatinum(II) [1,2]

Brief Reports and Preliminary Communications

Steven E. Vogl,* Mamdouh Moukhtar, and Barry H. Kaplan [3]

SUMMARY

Nine patients with advanced cancer of the uterine cervix, having failed radiotherapy, were treated with a combination regimen of parenteral methotrexate, bleomycin, and *cis*-dichlorodiammineplatinum(II). Eight of the patients had objective partial remissions lasting a median of 4 months. All patients improved subjectively. Five of the patients, however, had sudden unpredictable onset of severe myelosuppression accompanied by stomatitis. Such frequent severe toxicity was not observed in patients with other malignancies treated with this combination. It is likely that impaired excretion of methotrexate resulting from ureteral dysfunction led to increased toxicity. In future protocols for advanced cervical cancer, methotrexate will be replaced by other effective agents. The very high response rate (89%) suggests that similar but less toxic combinations may contribute substantially to the future effective treatment of advanced cervical cancer.

[Cancer Treat Rep 63:1005–1006, 1979]

In spite of the availability of effective cytologic screening, there are 20,000 new cases each year of invasive cancer of the cervix which result in 7400 deaths (1). Chemotherapy is used to treat patients who have metastases outside the pelvis or who have extensive pelvic disease which has persisted or recurred despite radiotherapy. Several drugs have shown some degree of activity (2). Most encouraging was a study of a combination of low doses of methotrexate (MTX) and bleomycin (BLM), with 12 of 20 patients responding (3). The median duration of survival for responders was 7.5 months. The present study is an attempt to improve results by adding a new agent, *cis*-dichlorodiammineplatinum(II) (DDP), to MTX and BLM.

At the time this chemotherapy protocol was designed, there was no evidence that DDP was active as a single agent in cancer of the uterine cervix. It has recently been shown that this agent has a high degree of activity in cervical carcinoma, with 11% complete (CR) and 33% partial (PR) remissions (4). The regimen we used, referred to as MBD (MTX, BLM, and DDP), is tolerable and effective in our hands for outpatient chemotherapy for advanced squamous cancer of the head and neck (5). In this disease, 61% of the patients had objective remissions, of which 22.5% were clinical CRs. The median duration of CR was 12 months. We elected to test the MBD regimen in cancer of the cervix, since the latter is histologically similar to head and neck cancer.

MATERIALS AND METHODS

Nine patients were treated with the MBD regimen. The median age was 56 years, with a range of 33–75 years. Three patients were totally bedridden, one was in bed for more than half the day, two were in bed for less than half the day, one was ambulatory with symptoms, and two were asymptomatic. The median time from initial diagnosis to start of chemotherapy was 40 months, with a range of 10–100 months. All patients had been treated with high doses of prior radiotherapy, but one patient had had this treatment interrupted by pelvic peritonitis thought to be related to a perforated uterus. Three patients had had prior major surgery, although this was palliative in two. The initial stage was IB in two patients, IIA in one, and IIIA in one. Sufficient data for proper staging are not available for the other five patients, all of whom received their initial treatment elsewhere. Sites of measurable disease included lymph nodes in four patients (supraclavicular, two; inguinal, one; and pelvic evaluable on computerized axial tomographic scan,

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one), lung lesions in three, pelvic mass in one, and lytic bone lesion with ipsilateral leg edema in one.

The chemotherapy consisted of 21-day cycles. On Days 1 and 15, MTX was given im at a dose of 40 mg/m^2. On Days 1, 8, and 15, BLM was given im at a dose of 10 units. On Day 4, DDP was given iv at a dose of 50 mg/m^2. This agent was given 30 minutes after starting a 2-hour infusion of 2 liters of 5% dextrose in 0.45% sodium chloride containing 10 mEq KCl/liter. Furosemide (40 mg) was given iv at the start of the infusion, and mannitol (12.5 g) was given iv just prior to the DDP.

Doses of MTX and DDP were reduced by 50% for wbc counts of 3000–4000/μl or platelet counts of 75,000–100,000/μl. These drugs were withheld if blood cell counts fell below these levels. MTX and BLM were withheld when stomatitis was present.

PR was defined as a 50% reduction in the estimated cross-sectional area of measurable sites of disease without evidence of worsening at any site and without deterioration of the general health of the patient. PR was considered terminated when new lesions appeared, when there was a 25% increase in the estimated cross-sectional area of the measurable lesion, or when there was a major deterioration in performance status or symptoms. Remission duration and survival were calculated from the start of treatment.

RESULTS

Eight (89%) of the nine patients treated had PRs. The ninth patient had marked reduction in pain and marked decrease in leg edema lasting 4 months. The site of measurable disease in this patient was a lytic lesion in the left ilium, which did not change. Remissions lasted a median of 4 months, with a range of 1.5–6. The median survival was 8 months (range, 4–14). Responses were accompanied by major improvements in performance status.

Five of the patients experienced sudden unpredictable onset of severe toxicity, sometimes after having tolerated one or more cycles of chemotherapy without incident. Two patients had nadir wbc counts of $< 1000/\mu l$, and three more had nadir wbc counts of $< 2000/\mu l$. Platelet counts of $< 25,000/\mu l$ were noted in two patients. Four of the five patients had stomatitis coincident with their myelosuppression. Three of these five patients had radiologically documented obstructive uropathy. In one of these patients, toxicity occurred shortly after loosening of a ureteral catheter, which led to hematuria and a mild degree of renal insufficiency. There were no deaths related to treatment.

Stomatitis was noted at some point in seven patients; it was mild in two, moderate in two, and severe in three. There was no azotemia among any patients in this series except that related to dehydration during periods of profound MTX intoxication (two patients). No patient had hearing loss. Nausea and vomiting related to DDP were moderately severe, and led one patient to refuse further therapy after four cycles in spite of a significant PR of her pelvic mass.

There was no evidence in this study, based on the temporal course of myelosuppression and stomatitis, that DDP in any way enhanced the toxicity of MTX.

DISCUSSION

The response rate of 89% (100% if a patient with marked subjective improvement is included) is quite high. All patients appeared to benefit from therapy. However, the present experience suggests that large intermittent doses of parenteral MTX should be administered with extreme caution to patients with advanced cancer of the cervix. It may be that minimal changes in ureteral caliber causing decreases in glomerular filtration rate too small to affect serum creatinine and BUN levels can markedly enhance the toxicity produced by standard doses of this drug, which are well-tolerated by patients with other diseases. Ureteral function can be impaired in this group of patients by direct tumor involvement or by scarring from prior surgery or irradiation. Perhaps more frequent administration of much lower doses of MTX, as was done in the study by Conroy et al (3), would avoid such toxicity.

Since responses observed in this study were not very long (median, 4 months), and since toxicity led five of the patients to spend an inordinate amount of their time in remission in the hospital, we would prefer to avoid further administration of MTX to this group of patients. In future studies in advanced cancer of the cervix, we intend to continue using BLM and DDP, and will add other effective agents, such as mitomycin C (6).

Should further study confirm the high response rate with similar, but less toxic regimens, combination chemotherapy should be tested in conjunction with radiation therapy for locally advanced cervical cancer.

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Phase II Evaluation of Chlorozotocin in Patients With Renal Cell Carcinoma [1,2]

Brief Reports and Preliminary Communications

Richard J. Gralla* and Alan Yagoda [3,4]

Chlorozotocin is a new water-soluble chloroethyl nitrosourea with high alkylating and low carbamoylating activities (1) that is now undergoing disease-oriented clinical trials at several treatment centers. Preclinical studies demonstrated activity in B16 melanoma and in L1210 and P388 leukemias. Myelosuppression, with thrombocytopenia being the most prominent manifestation, was found to be the dose-limiting toxicity in phase I trials (2–4). Because of reports of occasional objective responses in renal cell carcinoma with chlorozotocin in phase I studies (3,4) and reported activity of CCNU (5) in this disease, the present phase II study was initiated.

MATERIALS AND METHODS

Twenty-one patients with histologically confirmed unresectable renal cell carcinoma were entered in this study between June 1977 and February 1978. All patients had objectively measurable disease, a platelet count of at least 100,000/mm^3, a wbc count of at least 4000/mm^3, and a performance status (PS) (Karnofsky scale) of $\geq$ 50. The median age of the patients (15 males and six females) was 54 years (range, 28–71); the median PS was 70 (range, 50–90). Nine patients had received no prior therapy, five had been treated with radiation therapy, five had received chemotherapy, and two had received progestins.

Chlorozotocin (supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md) was given in equally divided doses for 5 consecutive days as a rapid iv injection. The initial total dose was 200 mg/m^2 for patients without prior chemotherapy who had a PS of $\geq$ 70. Patients who had received prior chemotherapy and had a PS of $\geq$ 70 were started at a dose of 175 mg/m^2, while those with a PS of 50 or 60 received an initial total dose of 150 mg/m^2. Courses were repeated every 6 weeks if the platelet count had returned to $\geq$ 100,000/mm^3 and if the wbc count had returned to $\geq$ 3500/mm^3. If no myelosuppression was observed, the subsequent course was given at a total dose that was 25 mg/m^2 higher than the previous course, not exceeding 200 mg/m^2. If the platelet count was $<$ 50,000/mm^3 or if the wbc count was $<$ 2000/mm^3 the next course of chlorozotocin was reduced by 25 mg/m^2.

Patients were considered evaluable 4 weeks after the initiation of chlorozotocin therapy. Therapeutic responses were defined as: *partial remission (PR)*—a $\geq$ 50% reduction of all measurable lesions for at least 1 month; and *minor response*—a $>$ 25% reduction of all measurable lesions, but less reduction than that required for a PR.

RESULTS

Therapeutic Responses

All patients received an adequate trial of chlorozotocin; however, no PRs occurred in these 21 patients. Minor responses were noted in three patients and consisted of regression (duration, 1, 2, and 2 months respectively) of pulmonary metastases in all patients. One of these patients had not received prior treatment, one had been treated with progestins, and one had received prior chemotherapy (hydroxyurea). All responding patients had a PS of $\geq$ 70. Median survival was 5 months for all patients and 6½ months for the three patients with minor responses.

Toxicity

Patients received a median of two courses of chlorozotocin (range, one to six courses). Thrombocytopenia was the most frequent dose-limiting factor. Table 1 shows the hematologic toxicity observed and the number of patients treated at each dose level. Two patients received platelet transfusions as a result of chlorozotocin-induced thrombocytopenia; however, severe hemorrhage did not occur. Cumulative hematologic toxicity was not observed and no patient experienced hepatic or renal dysfunction in association with chlorozotocin therapy. Nausea or vomiting was generally mild and occurred in $<$ 10% of the patients treated in this study.

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TABLE 1.—Hematologic toxicity in 21 patients with renal cell carcinoma treated with chlorozotocin

Total 5-day dose (mg/m^2)*	No. of—		Wbc nadir ($\times 10^3/mm^3$)			Platelet nadir ($\times 10^3/mm^3$)		
	Patients	Courses	Median	Range	Median Day	Median	Range	Median Day
150	3	5	3.8	3.3–7.1	41	89	24–130	31
175	10	19	3.6	1.8–4.9	44	62	20–212	28
200	12	21	4.4	1.0–7.0	38	57	14–182	29

*Chlorozotocin was administered as 5 equal daily doses.

DISCUSSION

No significant tumor regression occurred in the 21 chlorozotocin-treated patients. The treatment group included patients with a good PS who experienced myelosuppression secondary to chlorozotocin. In addition, 14 of 21 (67%) of the patients had received no prior hormonal or myelosuppressive chemotherapy. Because no PRs were achieved in this number of patients, it can be concluded that chlorozotocin is likely to have a true major response rate of $< 15\%$ ($P = 0.05$) (6). The drug would appear to have little therapeutic value in this schedule as a single agent in patients with advanced renal cell carcinoma.

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Proceedings of the Workshop on Amino Acid Imbalance in the Treatment of Cancer

The Upjohn Company

and

The National Cancer Institute

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Bethesda, Maryland

Introduction: Possibilities for Biochemically Rational Chemotherapy for Some Malignancies With Depleting Enzymes and Antimetabolites of Specific Amino Acids

Ladislav J. Hanka [1,2,*]

SUMMARY

A number of recent investigations have documented that, in several cancers, the malignant cells are no longer capable of synthesizing a specific amino acid, while the normal cells continue such biosynthesis at a normal rate. Enzymes degrading amino acids have demonstrated therapeutic value against such cancers. Significant progress was made recently in technology related to enzyme preparations. These are highly specific, have long half-lives, and can be chemically modified to eliminate their immunogenicity to man or animals. A practical in vitro system for detection of antimetabolites of different amino acids in natural materials was developed. It appears reasonable to expect that, in a specific malignancy, after the available pools of such deficient amino acids are exhausted by administration of the depleting enzyme, an additional, perhaps synergistic, effect could be obtained by utilizing an analog of such an amino acid. A brief review of the pertinent existing information will be presented in this communication.

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Information on Specific Amino Acids

L-*Asparagine.*—It has been reported that some leukemic cells have lost the ability to synthesize L-asparagine. The administration of L-asparaginase depletes the available pools of this amino acid and proves to be profoundly harmful to the leukemic cells, while normal cells, capable of synthesizing L-asparagine internally, are not seriously affected. The clinical value of L-asparaginase in the treatment of some leukemias is well-recognized (1,2). It would be reasonable to expect that such L-asparagine-starved leukemic cells would tend to assimilate analogs of L-asparagine with avidity, and that an increased therapeutic effect would result from such a combination. Several investigators have tested such combinations both in animal and human malignancies (3–6).

L-*Glutamine.*—Antagonists of L-glutamine (eg, azaserine and DON) have been tested extensively against tumors in animals and man in the past. In addition, several investigators have evaluated the use of L-glutaminase in the treatment of some malignancies (7–9). More recently, investigations were conducted to evaluate combination treatment with L-glutaminase and antimetabolites of L-glutamine such as DON or azaserine (8,9) or L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (NSC-163501) (10).

L-*Tyrosine.*—An increased requirement for L-tyrosine (and to a lesser degree, for L-tryptophan) was reported in cells of melanoma by Pawelek in 1976 (11). Meadows et al reported that L-tyrosine phenolyase inhibited significantly B16 melanoma in mice (12).

L-*Phenylalanine.*—Abell et al reported that an L-phenylalanine-degrading enzyme inhibited the growth of murine leukemia in vivo (13). Elmer et al reported in vivo activity of this enzyme against B16 melanoma (14).

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L-*Tryptophan*.—Purification of a new L-tryptophan-metabolizing enzyme with antitumor activity in mice was reported recently by Roberts and Rosenfeld (15).

L-*Methionine*.—The therapeutic potential in animal tumors of an L-methionine-degrading enzyme was described in 1973 by Kreis and Hession (16).

L-*Cystine*.—Foley et al have reported (17) that some human malignant cells have an unusual and specific requirement for L-cystine. Uren and Lazarus (18) reported that L-cystine-cysteine-degrading enzymes inhibited the growth of leukemic cells in vitro. Thus, it might be desirable to explore the potential of a combination treatment with such L-cystine-degrading enzymes and analogs of that amino acid.

L-*Threonine*.—Greenfield and Wellner reported recently (19) that certain leukemic cells were more sensitive to L-threonine deprivation than normal cells. They suggested that L-threonine deaminase should be evaluated for its possible antitumor effect in vivo.

L-*Serine*.—There are published data (20) suggesting possible utility of L-serine dehydratase in leukemia.

L-*Histidine*.—The possible usefulness of an enzyme degrading L-histidine has been suggested by Holcenberg and Roberts (21).

L-*Arginine*.—There are reports on the effect of L-arginase against animal tumors (21,22). However, additional work is indicated to establish the real potential of this enzyme.

This whole area is in a stage of fast progress. In 1974, Cooney and Rosenbluth (23) reviewed the antineoplastic activity of several amino acid-degrading enzymes. An up-to-date review of this field was recently presented by Holcenberg and Roberts (21).

Possible Ways to Eliminate the Immunogenic Effect of the Enzyme Preparations

Recent reports by Abuchowski et al (24) indicate that it might be possible to eliminate, or at least strongly reduce, the immunogenic effects of the enzymes used in therapy. Development of such techniques offers considerable promise in extending the prospects of such therapy.

Methods for Discovery of Antimetabolites of Specified Amino Acids

In the early 1960s, an in vitro screen for detection of antimetabolites was developed by The Upjohn Co and was described by Hanka (25,26). Antimetabolites of amino acids are found much more frequently than, for instance, those of purines or pyrimidines. Scannell and Pruess published a review of the naturally occurring amino acid antimetabolites in 1974 (27). Thus, a number of antimetabolites of the desirable amino acids are available. Undoubtedly, other and possibly better antimetabolites can and should be found either by isolation from biologic material or by chemical synthesis.

Early studies using a combination of a depleting enzyme with an antagonist of that specific amino acid were limited to L-asparaginase-glutaminase. Even though the animal data were encouraging, the method did not gain clinical use. The fast progress in this field, however, might offer new possibilities.

More specific and longer-lasting enzyme preparations are now available; apparently it is possible to eliminate their potentially immunogenic properties by proper treatment prior to application. Historically, one of the roadblocks to faster success in the chemotherapy for cancer was that very few biochemical differences between malignant and normal cells were established. Such differences now seem to exist in the area of amino acid metabolism.

Considering all these developments, a special workshop was convened at the National Cancer Institute on May 23, 1978. A number of invited speakers reviewed the existing state of knowledge in several areas pertinent to this field of research. The proceedings of the workshop are presented in this issue of *Cancer Treatment Reports*. The sequence of the papers will largely follow the organization of the workshop as outlined below:

1. Past clinical observations suggesting that amino acid manipulations are relevant to cancer treatment.
2. Deficiency of specific amino acids in some malignancies and therapeutic value of the corresponding depleting enzymes.
3. Antitumor activity of some new amino acid antimetabolites used alone or in combination with depleting enzymes.
4. Advances in enzyme preparation.
5. Methods of detecting amino acid antimetabolites.

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Lessons From the Study of Induced Alterations in Amino Acids in Patients With Cancer

James F. Holland* and Takao Ohnuma[1]

SUMMARY

The sensitivity of the neoplastic cell to amino acid deprivation shows considerable variation. The responsiveness of human leukemic cell cultures to L-asparaginase indicates that those of T cell origin are much more sensitive to the action of this L-asparagine-depleting enzyme than those of B cell origin. A cautionary note is raised concerning amino acid analogs. Minor changes such as substituting selenium for sulfur to make seleno-L-methionine rather than L-methionine may lead to considerable differences in their respective metabolic pools in vivo. A systematic survey of amino acid requirements of human neoplasms is desirable before designing new analogs.

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L-ASPARAGINASE

The recognition by Kidd of an antileukemic effect upon administering guinea pig serum to mice, and the subsequent demonstration by Broome that this was due to L-asparaginase, is a classic instance of the unanticipated (1). L-Asparaginases that have been used in man for acute leukemia include the guinea pig L-asparaginase by Dolowy, and that of a South American rodent, the agouti. Earlier, we studied the effects of chicken liver L-asparaginase against mice bearing the 6C3HED lymphoma, but it was cleared too rapidly from the blood to have therapeutic effects (2). The field opened greatly when Mashburn isolated L-asparaginase from *Escherichia coli*. It was an extremely active drug in mouse leukemia, particularly the 6C3HED lymphoma (1).

L-Asparaginase was tested in canine lymphomas by Old and colleagues, then in man by Oettgen et al, and subsequently by many other investigators (1). When compared to its extraordinary effects against the 6C3HED lymphoma, the activity of the drug in man was disappointing. In vitro incubations were made and measurements were taken of the incorporation of uridine, L-valine, or L-lysine into the macromolecules of this mouse lymphoma and acute lymphocytic leukemia (ALL) and acute myelocytic leukemia (AML) in man in the presence or absence of L-asparaginase (fig 1). The correlation of in vitro prediction and in vivo therapeutic result was good in the 6C3HED lymphoma, but poor in man (3). L-Asparaginase was first used in patients who had relapsed from remissions induced with vincristine and prednisone that had been maintained with antimetabolites. L-Asparaginase alone, or L-asparaginase in combination with vincristine and prednisone, with or without daunorubicin, led to a second remission. There are still debates concerning the optimal L-asparaginase dose schedule. We found that weekly administration of L-asparaginase had a higher frequency of anaphylaxis than daily administration. Hill reported that high-dose L-asparaginase eliminates anaphylaxis altogether (1). Other investigators have used im administration of the drug on a three times weekly schedule with success (1).

Recently, we reported differential sensitivity to L-asparaginase among human cells of the lymphoid series. T lymphocytes are susceptible to L-asparaginase at much lower concentrations than B cells (4) (fig 2). This is of considerable interest since large and small cell lymphosarcomas are now recognized to be primarily derivative of B cells; they are quite insensitive to L-asparaginase. ALL, which is recognized to be primarily a null cell disease, would presumptively be pre-T cell leukemia from these data. Common type and T cell ALL are susceptible to L-asparaginase. AML is also occasionally susceptible

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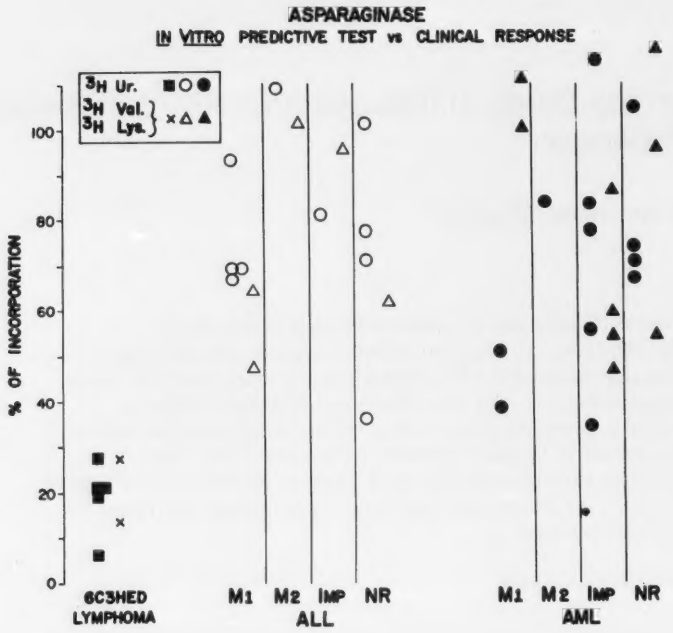

FIGURE 1.—Effect of L-asparaginase on incorporation of an RNA or protein precursor into macromolecules of malignant cells in vitro expressed as % of control incorporation. The significant depression seen in 6C3HED lymphoma was not observed in ALL or in AML. There is no correlation between L-asparaginase inhibition and the best response the patient achieved with L-asparaginase treatment. M1 = reduction of marrow blast cells to ≤ 5% with restoration of normal hematopoiesis; M2 = reduction of blast forms and leukemic cells to ≤ 25%; IMP = lesser improvement; and NR = no response (*from* ref 3).

to L-asparaginase, but at a much lower frequency than ALL. We have reported on one patient who did indeed enjoy a remission after a single high dose of L-asparaginase (5). This biochemical distinction of B cells and T cells in vitro correlates with sensitivity to only two other drugs: cytosine arabinoside (ara-C) and 5-fluorouracil. T cell lines are nearly 100-fold more sensitive to ara-C than B cell lines (fig 3) (6) and B cell lines are about 20-fold more sensitive to 5-fluorouracil than T cell lines. About a dozen other drugs that are commonly used in cancer show no selectivity between B and T cell lines.

The use of L-asparaginase has become widespread in childhood ALL. The Cancer and Leukemia Group B recently showed that a variable as small as when in the course of treatment L-asparaginase is administered can make a significant difference in the effect (7). A difference in the duration of complete remissions and in the mortality associated with L-asparaginase administration was observed. Children were randomly allocated to receive vincristine and prednisone alone, or to receive L-asparaginase before, simultaneously with, or after 3 weeks of vincristine and prednisone. The remission frequency was not altered significantly, but differences in remission duration were significant (fig 4). Furthermore, if one takes, as a baseline, the death rate during induction from vincristine and prednisone, then simultaneous or antecedent administration of L-asparaginase leads to a significantly higher death rate. This may be due to alterations in the metabolism of vincristine and prednisone when L-asparagine is deficient in the liver and synthesis of metabolizing enzymes is changed by the enzyme. L-Asparaginase given after completion of the vincristine and prednisone administration is not associated with the augmented vincristine toxicity and death rate seen in the antecedent and simultaneous groups. A possible explanation of increased effect is that there are changes in tumor kinetics following early vincristine and prednisone administration because the tumor is now smaller. L-Asparaginase alters L-asparagine availability for the brain leading to central nervous system dysfunction. Major alterations in psychologic and motor factors occur in extreme instances. Rescue by L-asparagine infusion has been described (3,5,8).

L-Asparaginase leads to anaphylaxis in a small number of patients. We first reported the use of *Erwinia carotovora* L-asparaginase without cross reaction in four patients who had undergone frank anaphylaxis from *E. coli* enzyme (9).

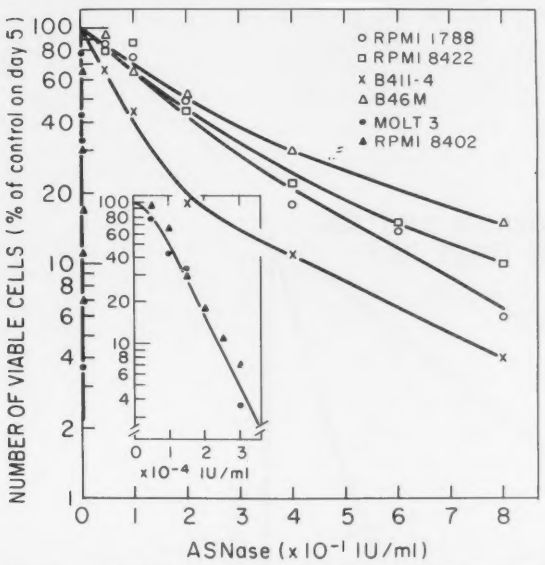
INHIBITION OF CELL GROWTH BY ASNase
NUMBER OF VIABLE CELLS (% of control on day 5)
ASNase (x 10⁻¹ IU/ml)
o RPMI 1788
□ RPMI 8422
x B411-4
Δ B46M
• MOLT 3
▲ RPMI 8402
x10⁻⁴ IU/ml

FIGURE 2.—Inhibition of cell growth of 2 human T cell lines (filled symbols) and four B cell lines (open symbols) by *E. coli* L-asparaginase (ASNase). Culture flasks contained an initial viable cell concentration of 2×10^5 cells/ml of RPMI-1640 medium with 10% dialyzed fetal calf serum plus different concentrations of the enzyme. After 5 days' growth, viable cells were counted and plotted, assuming the viable cell concentration in the enzyme-free medium to be unity. 4 lines of B cells show sensitivity to L-asparaginase markedly different from 2 lines of T cells (filled symbols overlying Y axis). T cells show extreme sensitivity to L-asparaginase at 1000-fold lower concentrations (inset box). The mechanism of inhibition of L-asparaginase on B cells at high concentrations is unknown and not related to its L-asparaginase or L-glutaminase activity (*from* ref 4).

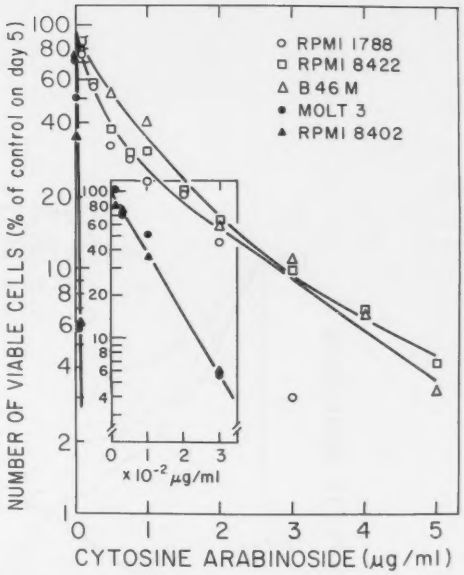

FIGURE 3.—Differential inhibitory effects of ara-C on 2 human T cell lines (filled symbols) and 3 B cell lines (open symbols) in culture (*see* legend of fig 2 for the experimental procedure). B cells show sensitivity to ara-C markedly different from T cells (filled symbols near Y axis). T cells show sensitivity to ara-C at 100-fold lower concentrations (inset box) (*from* ref 6).

AMINO ACID ANALOGS

[^{75}Se]seleno-L-methionine is a gamma-emitting amino acid, widely accepted as an analog of L-methionine. Even though it resembles L-methionine, it is not a true analog. Mice were total body-labeled over several weeks of feeding on a diet to which [^{75}Se]seleno-L-methionine had been added. They were then randomized to be fed a complete diet, a complete diet plus gram quantities of supplementary L-methionine, a complete diet plus microgram quantities of seleno-L-methionine, or a protein-free diet. The decay curves for the total body counts show substantially greater loss in the presence of seleno-L-methionine, indicating that seleno-L-methionine is not in the same pool with L-methionine (fig 5) (10).

In cancer patients labeled iv with L-[^{35}S]methionine and [^{75}Se]seleno-L-methionine simultaneously, and given L-asparaginase, the curves for albumin biosynthesis before and after L-asparaginase are different. Furthermore, the kinetics are different for the two isotopic amino acids (fig 6).[2] In both subjects the L-[^{35}S]methionine incorporation after L-asparaginase decreases slightly in the first one-half hour and fails to exceed initial levels by 90 minutes. This is in contrast to [^{75}Se]seleno-L-methionine, the incorporation of which increases consistently after administration, despite L-asparaginase. In subject K (fig 6) [^{75}Se]seleno-L-methionine incorporation appears totally uninfluenced in the first 90 minutes, whereas in subject P (fig 6) it is somewhat retarded. We also have unpublished data on animals triply labeled with [^{75}Se]seleno-L-methionine, L-[^{35}S]methionine, and either L-[^{3}H]lysine or L-[^{3}H]valine. The rate of protein synthesis as measured by L-[^{3}H]valine or L-[^{3}H]lysine incorporation remained constant. Reciprocal dilution of the other two isotopic amino acids, either with seleno-L-methionine or with L-methionine, demonstrated that these two amino acid species are not in the same pool.

[^{75}Se]seleno-L-methionine, although it labels for protein biosynthesis, does not label for the same protein biosynthesis in which L-methionine participates. This point is

[2]Holland JF, Arakawa W, and Kufe D. Unpublished observations.

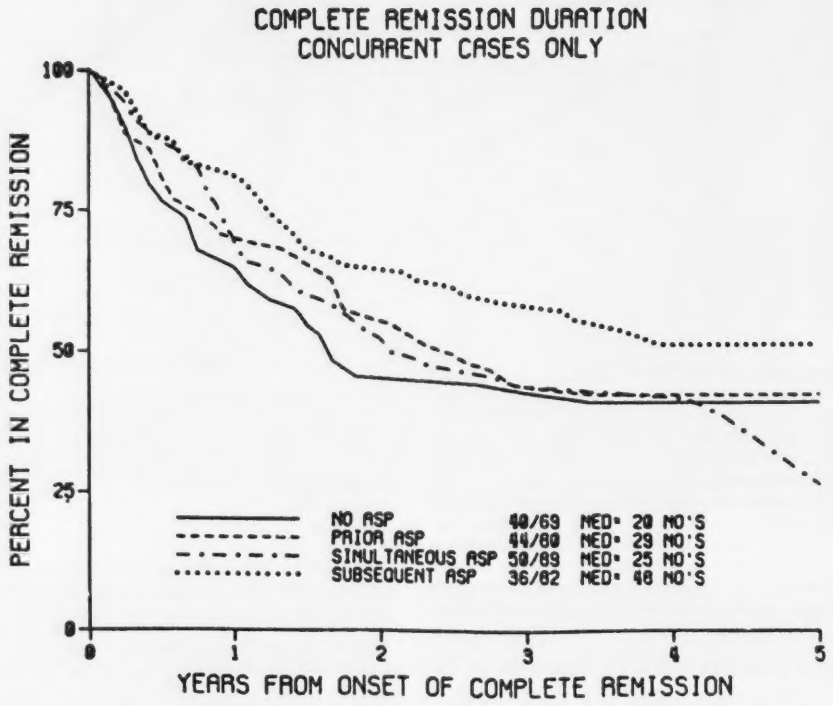

FIGURE 4.—Children with ALL studied in a comparative randomized trial were induced into remission with vincristine and a corticosteroid (no L-asparaginase [ASP]) or with these 2 drugs plus iv L-asparaginase at a dose of 1000 IU/kg/day × 10 days. Prior, simultaneous, and subsequent L-asparaginase describe administration with respect to the 21-day course of vincristine and a corticosteroid. Patients were randomly assigned; Nos. = No. of failures/sample size at risk and median mos of remission duration. There have been 36 relapses among 82 patients at risk for a median remission duration of 48 mos in the subsequent L-asparaginase group (*from* ref 7).

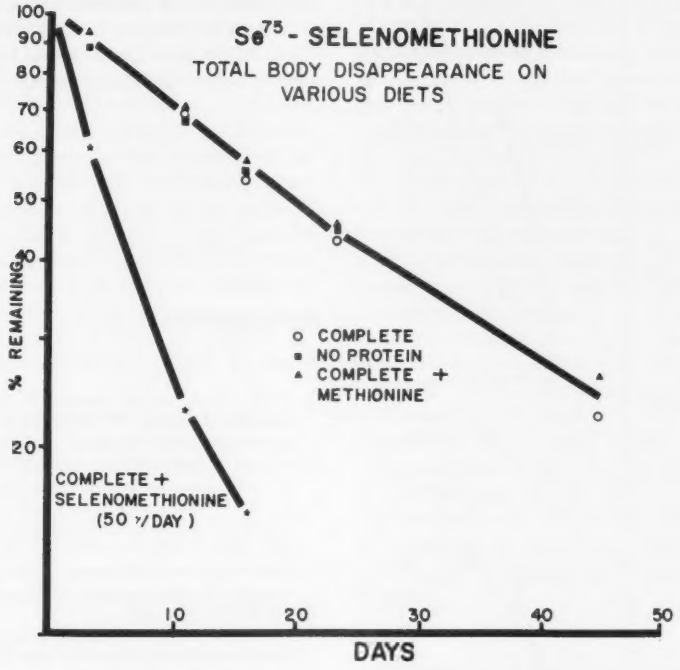

FIGURE 5.—Rate of loss of total body [^{75}Se]seleno-L-methionine is identical in normal Swiss mice (prelabeled by continuous feeding) that received (*a*) a diet complete in protein, (*b*) a diet with no protein, or (*c*) a complete diet plus added L-methionine. Microgram quantities of seleno-L-methionine added to the complete diet accelerated [^{75}Se]seleno-L-methionine loss, however, demonstrating that this isotopic amino acid is in a metabolic pool distinct from L-methionine (*from* ref 10).

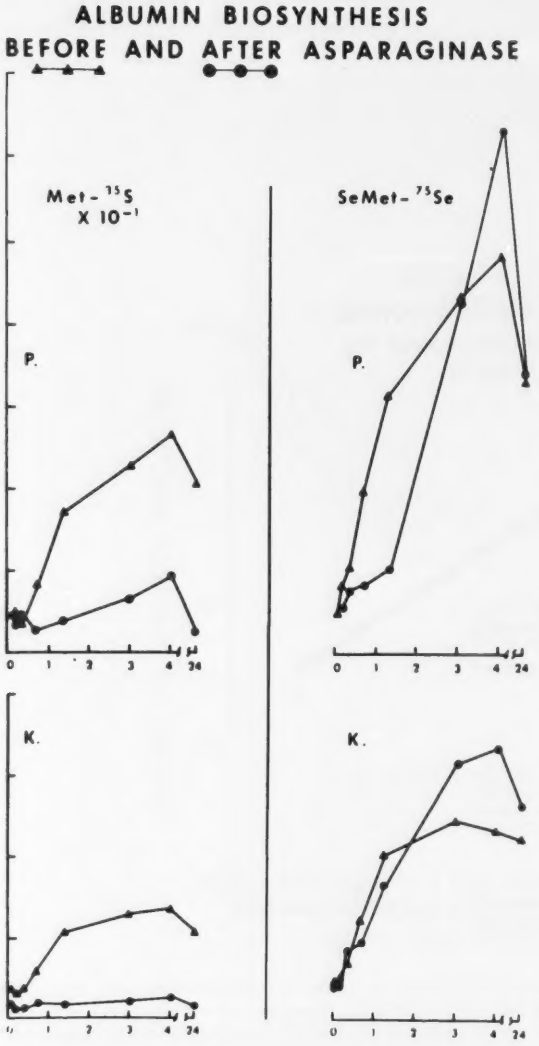

FIGURE 6.—2 patients were injected simultaneously with 100 microcuries of L-[^{35}S]methionine (Met-^{35}S) and 25 microcuries of [^{75}Se]seleno-L-methionine (SeMet-^{75}Se). Incorporation into isolated albumin was determined for each isotope after appropriate correction for background and for gamma radiation during beta detection. Abscissa = time in hrs; ordinate scale = unnumbered units representing counts/min/mg of albumin $\times 10^{-2}$ for [^{75}Se]seleno-L-methionine. Data for L-[^{35}S]methionine are further reduced by 10^{-1} thus representing 10^{-3}. Baseline kinetics are closely similar for the 2 amino acids for both patients (P and K). After L-asparaginase, L-[^{35}S]methionine incorporation into human albumin is sharply decreased but that of [^{75}Se]seleno-L-methionine is not. We conclude that seleno-L-methionine and L-methionine are in different metabolic pools.

raised as a cautionary note, insofar as this symposium deals with amino acid analogs which may have unique effects. These analogs should not necessarily be considered as true analogs of the parent amino acid after which they are named. However similar their structure appears to be to the model natural amino acid, their true function must be identified as if they were any new drug.

A study of amino acid requirements of human leukemic cells identified several which were essential and many which were not (11). A systematic survey of other human neoplasms could demonstrate other spectra of essential amino acids which were tissue-specific or tumor-specific. Such information about amino acid requirements is desirable before designing analogs.

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Achromobacter L-Glutaminase-L-Asparaginase: Human Pharmacology, Toxicology, and Activity in Acute Leukemias[1]

A. S. D. Spiers[2,*] and H. E. Wade[3]

Enzymes that hydrolyze L-asparagine to L-aspartic acid and ammonia, L-asparaginases, occur naturally in the sera of certain *Cavioidea* rodents, and are elaborated by several bacterial species. L-Asparaginases are cytotoxic to murine lymphomas both in vitro (1) and in vivo (1,2). The enzymes produced by *Escherichia coli* and *Erwinia carotovora* are effective agents for the induction of remissions in human acute leukemias, particularly acute lymphocytic leukemia (ALL), although a few cases of acute myeloid leukemia (AML) have also responded favorably (3–7). Problems of sensitization to bacterial proteins have ensured a clinical role for both of these L-asparaginases, since their lack of immunologic cross-reactivity enables the administration of the alternative enzyme to patients who have developed allergy to one of them.

The usefulness of L-asparaginases in ALL prompted a search for antitumor activity among other enzymes that degrade amino acids. Roberts et al (8) described an L-glutaminase-L-asparaginase isolated from a strain of *Achromobacteraceae*. The organism was subsequently characterized as *Acinetobacter glutaminasificans* and the L-glutaminase-L-asparaginase enzyme is now called AGA. It was found that AGA has L-glutaminase-L-asparaginase activity at a ratio of 1.2:1, and was cytocidal in vitro to cells from patients with chronic lymphocytic leukemia (CLL), plasma cell leukemia, and AML (9). AGA also inhibited the mitogenic response of normal lymphocytes to phytohemagglutinin in vitro, and was active at much lower concentrations than *E. coli* L-asparaginase, suggesting that the inhibitory effect on blastogenesis was due to the L-glutaminase activity, rather than the L-asparaginase hydrolyzing property, of the enzyme (10). Schrek and co-workers (11) showed that acute leukemia cells generally required both L-glutamine and L-asparagine for survival in vitro, but that CLL cells were killed by L-glutamine deprivation and not by L-asparagine deprivation. The cytotoxic effects of L-glutaminase could not be mimicked by the addition of ammonia and sodium L-glutamate to media containing L-glutamine without L-glutaminase (9), ie, cell-kill apparently is due to depletion of L-glutamine per se and not to the accumulation of the products of its enzymatic hydrolysis. That L-glutaminase possesses antitumor effects quite separate from its L-asparaginase activity was confirmed by Schmid and Roberts (12), who demonstrated cytocidal effects when three L-asparaginase-resistant ascites tumors were treated with L-glutaminase-L-asparaginase enzymes from *A. glutaminasificans* (AGA) and *Pseudomonas aureofaciens*.

As part of a program for development of enzymes with antitumor activity, an L-glutaminase-L-asparaginase enzyme was produced at the Microbiological Research Establishment from *Achromobacter* 257. It showed promising antitumor activity both in vitro and in animals; this prompted a phase I study in man. The usefulness of the L-asparaginases in human acute leukemias and the in vitro antileukemic activity of the *Acinetobacter* enzyme (9) prompted us to conduct the phase I study in patients with leukemia in the hope of demonstrating antitumor activity while studying the human pharmacology of the enzyme.

PATIENTS, MATERIALS, AND METHODS

Patients

Six patients with ALL and four with AML received the *Achromobacter* enzyme (table 1). All were in relapse after one or more remissions, and, in all, the leukemia had become resistant to standard antileukemic therapy and to an aggressive eight-drug regimen (13). Resistance to L-asparaginase was documented in all ALL patients. Informed consent was obtained from patients or their parents. The indices measured before, during, and after the administration of the *Achromobacter* amidohydrolase were: hemoglobin, reticulocyte and platelet counts, total and differential leukocyte counts, serum electrolytes, BUN, creatinine, urate, SGOT, cholesterol, albumin, globulin, calcium, phosphate, alkaline phosphatase, bilirubin, and blood glucose. Blood pH and blood gases were measured in some patients, and the bone marrow was examined if leukemic cells were absent from the blood.

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TABLE 1.—Patients with refractory acute leukemia who received *Achromobacter* L-glutaminase

Patient No.	Age/ sex	Diagnosis	Resistance to L-asparaginase	Dose of L-glutaminase (IU/m²/day)	Schedule	No. of days	Antileukemic effects
1	32/M	ALL	+	3500	Daily iv	14	Blast cell count rose from 0 to 4000/μl in 5 days after cytotoxic therapy but remained 0 for 14 days when L-glutaminase followed the cytotoxic therapy; blast cells rose promptly after L-glutaminase
2	8/M	ALL	+	4000	12-hr sc	20	Blast cell count fell from 11,000 to 2500/μl in 6 days, then rose despite continuing L-glutaminase
3	17/M	ALL	+	12,000	12-hr iv	14	Blast cell count fell from 2900 to 120/μl; marrow blast cells fell from 75% to 50%
4	17/M	ALL	+	20,000	Iv infusion	7	Blast cell count fell from 135,000 to 63,000/μl in 6 days; subarachnoid hemorrhage then developed
5	40/F	AML	+	20,000	Iv infusion	3	Treatment stopped on 3rd day due to onset of severe infection; response not evaluable
6	14/M	ALL	+	20,000	Iv infusion	28	Regression of 8-cm splenomegaly; blast cell count fell from 3300 to 0/μl
7	7/M	AML	?	15,000	Iv infusion	12	Blast cell count fell from 17,000 to 0/μl in 7 days; became pancytopenic
8	27/M	ALL	+	10,000	Iv bolus	1	Test dose only
9	68/M	AML	?	20,000	Iv infusion	4	Blast cell count fell from 8000 to 240/μl in 4 days; became acidotic
10	25/M	AML	?	17,000	Iv infusion	5	Blast cell count fell from 23,000 to 3100/μl in 7 days; became acidotic and hyperglycemic
				8000		5	Blast cell count fell from 15,000 to 8400/μl in 8 days; became acidotic and hyperglycemic

L-Glutaminase

The enzyme was produced by fermentation and purified to a homogeneous preparation containing 150 IU/mg of protein. It was supplied as a sterile lyophilized powder in ampules of 1000 or 2000 IU. The powder was readily soluble in physiologic saline and the reconstituted solution was nonpyrogenic. The molecular weight of the enzyme is 135,000 (± 10,000) and isoelectric focusing on polyacrylamide gel indicates an isoelectric point of pH 8.7. The enzyme shows about 20% greater activity toward L-glutamine than toward L-asparagine and expresses a Km of the order of 0.002–0.004 mM toward both amino acids. Thus, the Microbiological Research Establishment enzyme resembles AGA (8,14) in expressing similar activities toward L-glutamine and L-asparagine and having very high substrate activities. At a concentration of 20 mM, D-glutamine and D-asparagine are degraded at 35% and 26% respectively of the rate at which the L-isomers are hydrolyzed. In the context of cancer treatment, it was recognized at an early stage that a potential immunologic hazard would exist if the *Achromobacter* L-glutaminase-L-asparaginase had immunogenic determinants in common with the two L-asparaginases already in clinical use. Exploratory tests compared its serologic specificity to that of *E. coli* L-asparaginase (Merck Sharp & Dohme, West Point, Pa) and *Erwinia* L-asparaginase (Microbiological Research Establishment). *Achromobacter* L-glutaminase-L-asparaginase did not react with antisera to either of the L-asparaginases or with an antiserum to AGA. However, an antiserum to the *Achromobacter* enzyme partially inhibited both it and AGA. These studies suggested that immunologic problems were unlikely to arise if treatment with *Achromobacter* L-glutaminase-L-asparaginase followed treatment with either of the bacterial L-asparaginases in clinical use.

Blood Analyses

For pharmacokinetic studies, single iv bolus doses of the *Achromobacter* L-glutaminase-L-asparaginase (6000–10,000 IU/m² of body surface area) were administered. Blood samples taken before and at intervals after this dose were placed into ethylenediaminotetra-acetate tubes and frozen at the bedside in solid CO_2, then stored at −20°C. Immediately after thawing, one volume was diluted in 0.05 M sodium borate buffer, pH 8.5, for L-glu-

taminase assay (15), and a second volume (0.5 ml) was diluted with an equal volume of protein precipitant (2.5% sulfosalicylic acid in 0.5 M lithium sulfite) and centrifuged. The amino acid composition of the supernatant was analyzed (16) on a Locarte analyzer using L-norleucine as an internal standard. Thus, we determined the blood half-life of the enzyme and the duration of depletion of blood L-glutamine and L-asparagine after a single iv dose. In patients who received iv infusions of the drug, blood L-glutaminase, L-glutamine, L-glutamate, L-asparagine, and L-aspartate were measured at intervals during therapy.

Administration of L-Glutaminase

For pharmacokinetic studies, single iv bolus doses were given. In our preliminary studies of the antileukemic effects of the enzyme, four schedules were used: (*a*) daily iv bolus injections; (*b*) twice daily sc injections; (*c*) twice daily iv injections; and (*d*) continuous infusions of L-glutaminase in physiologic saline. When the blood half-life of the drug was found to be short, schedule (*d*) was used in all subsequent studies in order to obtain the maximum effect from the small supplies of L-glutaminase available.

RESULTS

Blood Half-Life

As previously reported (17), the blood half-life of the first batch (G1) of enzyme tested was about 80 minutes. After iv bolus doses, the pattern of decline in blood levels suggested a one-compartment distribution. In later studies with batch G4 of enzyme, which was shown by molecular weight determinations to have undergone some polymerization during preparation, the blood half-life was 115–120 minutes. With batch G4, the initial half-life (period 5–240 minutes) was 120 minutes, but later (period 240–1440 minutes) it approached 4 hours. We are currently working with another polymerized batch (G7), and further half-life determinations will be made.

Blood Amino Acids

The effects on blood levels of amino acids clearly were schedule-dependent. One patient (No. 1) received daily iv bolus injections beginning with 25 IU/m^2 daily, and reaching a maximum of 3500 IU/m^2 daily for 13 days; depletion of L-glutamine and L-asparagine was not observed in any blood sample. Another patient (No. 3) received 6000 IU/m^2 twice daily by iv injection; L-glutamine and L-asparagine were undetectable in the blood 90 minutes after injection, but returned to pretreatment levels by 12 hours. In patients 4 and 5, L-glutamine and L-asparagine were undetectable in the peripheral blood 5 minutes after single iv injections of 10,000 IU/m^2; depletion persisted at 2 hours. Another patient (No. 6) received 20,000 IU/m^2 of L-glutaminase each day by continuous iv infusion; L-glutamine and L-asparagine were always undetectable in the blood throughout the 28 days of L-glutaminase administration. Thus, it appears that single bolus injections of 6000–10,000 IU/m^2 of this L-glutaminase produce rapid and complete depletion of blood L-glutamine and L-asparagine, and that this state persists for at least 2 hours but is recovered by 12 hours. More detailed studies of the rates of reappearance of these amino acids in the blood after single doses of the L-glutaminase by a variety of routes are in progress.

Toxicity

No toxic effects occurred in patient 2, but the other nine patients were nauseated and five vomited. Vomiting occurred most often after initial iv loading doses of L-glutaminase; it was infrequent during continuous maintenance therapy by iv infusion. Nausea usually abated after a few days of treatment, although some patients experienced mild nausea throughout the period of L-glutaminase administration. It should be noted that all ten patients were ill with near-terminal acute leukemia, had received much prior cytotoxic therapy, and in many cases were nauseated before L-glutaminase therapy was begun. Drowsiness was observed in patients 7 and 10, and confusion was evident in patients 5, 9, and 10. In patient 5, confusion was associated with infection and meningeal leukemia and in patients 9 and 10 there was concurrent acidosis. Thus, it is uncertain whether L-glutaminase therapy per se produced mental confusion. No patient developed skin rash, urticaria, itching, bronchospasm, hypotension, or other manifestations of allergy. There was no gross disturbance in most of the biochemical indices studied. In one patient (No. 6) who received L-glutaminase continuously for 8 days, there was moderate depression of serum urate, bicarbonate, cholesterol, SGOT, and calcium levels, with an increase in serum phosphate concentration. The serum albumin level fell to 2.8 g/100 ml by the 10th day, but rose into the normal range by the 17th day. Hypouricemia also occurred in patients 4 and 10. Serum calcium levels were depressed and serum phosphate levels rose in patients 7 and 10; patient 7 also showed low SGOT levels in the blood. Hyperglycemia occurred in patients 4 and 10, but in patient 4, the biochemical change was preceded by a subarachnoid hemorrhage and hyperglycemia therefore could not with certainty be attributed to L-glutaminase. Metabolic acidosis occurred in patient 4 (after the subarachnoid hemorrhage) and in patients 9 and 10. Neutropenia, possibly attributable to L-glutaminase, occurred in five patients, and the platelet count was depressed in two.

Several patients were neutropenic and thrombocytopenic, and were receiving repeated platelet transfusions before the administration of L-glutaminase; therefore, myelotoxicity could not be assessed. Overall, the toxic effects were comparable with those of other treatments for acute leukemia. Treatment was interrupted because of toxicity in only two patients, who developed metabolic acidosis attributable to L-glutaminase which was reversed by discontinuing the enzyme and administering sodium bicarbonate iv.

Patient 9

Patient 9 was a 68-year-old man with AML in relapse after an initial remission; his leukemia failed to respond to aggressive reinduction therapy with conventional agents. L-Glutaminase (20,000 IU/m^2/day) was given by continuous infusion and the peripheral blood blast cell count fell from 7900 to 240/μl in 96 hours. He then developed metabolic acidosis with a blood pH of 7.3 and Kussmaul respiration; this was corrected in 12 hours by discontinuing L-glutaminase and administering iv sodium bicarbonate. The blast cell count rose to 8400/μl in the 4 days after discontinuing the drug. L-Glutaminase was then reinstituted at a dose of 5000 IU/m^2/day by daily sc injection. Over the next 4 days, the blast cell count rose to 24,000/μl and other antileukemic therapy was begun. It was uncertain whether true refractoriness to L-glutaminase had occurred or whether the dose was simply inadequate.

Patient 10

Patient 10 was a 25-year-old man who had had AML for 2 years. He had had multiple relapses and had become resistant to all conventional drugs as well as to 5-azacytidine. He received L-glutaminase (17,000 IU/m^2/day) by continuous iv infusion. The blast cell count fell from 22,700 to 3100/μl in 7 days, but by the fifth day he had become confused and developed a compensated acidosis (pH 7.52; bicarbonate, 14.5 mEq/liter; pCO_2,18) with hyperglycemia (blood glucose, 620 mg/100 ml), but no ketosis. The drug was discontinued on the fifth day and by the ninth day the blast cell count was 14,700/μl. L-Glutaminase was reinstituted by continuous infusion at a dose of 8000 IU/m^2/day, and the blast cell count fell to 8400/μl 8 days later. L-Glutaminase was discontinued on the fifth day of this second course of treatment because, even at the lower dose, mental confusion reappeared together with compensated acidosis (pH 7.4; bicarbonate, 14 mEq/liter; pCO_2, 22) and hyperglycemia (blood glucose, 680 mg/100 ml). During this episode, his serum calcium fell to 7.8 mg/100 ml with a phosphate level of 6.8 mg/100 ml. Serum amylase, bilirubin, SGOT, BUN, and creatinine were repeatedly normal, while the serum uric acid fell to 2.0 mg/100 ml. The second episode of confusion resolved in 24 hours with discontinuation of L-glutaminase and administration of bicarbonate, potassium, and insulin. From the fourth day of discontinuing L-glutaminase, his blast cell count rose progressively and other therapy was begun.

A very specific toxic effect was observed in patients who received an initial iv bolus dose of L-glutaminase. Approximately 2 minutes later, they experienced transient mental confusion, complained of difficulty in breathing, and vomited. Urticaria, bronchospasm, and hypotension were not observed, and in every patient the symptoms disappeared in 2–5 minutes without treatment. Such reactions were not seen with second or subsequent doses of L-glutaminase, and therefore seem most unlikely to be allergic in nature. Since our results show that, after the injection of L-glutaminase, there is very rapid hydrolysis of the entire blood content of L-glutamine and L-asparagine, it is possible that transient, marked rises in blood L-glutamate, L-aspartate, or ammonia may explain these disturbing reactions, which could be mistaken for anaphylaxis. We have occasionally seen a similar reaction after an initial iv injection of L-asparaginase. In later studies, when L-glutaminase has been started by slow iv infusion without a bolus dose, this disturbing reaction has not occurred.

Antileukemic Effects

Despite lack of knowledge of the optimum dose and schedule of administration, and the deliberate selection of patients with advanced acute leukemia refractory to multiple drugs, the antileukemic activity of L-glutaminase was convincingly demonstrated. Five of the six patients with ALL received repeated doses of L-glutaminase, and all five showed some response. All four patients with AML received repeated doses and three responded; in patient 5, the drug was discontinued on Day 3 due to the onset of severe infection. We observed symptomatic improvement, regression of splenomegaly, reduction in the circulating blast cell count, and a decreased proportion of blast cells in the bone marrow. No patient had complete remission but all were in a late stage of their disease (a performance status of 3 or 4) and had shown progressive deterioration despite conventional antileukemic therapy. In this preliminary study it was not possible to administer L-glutaminase to patients with less unfavorable prognostic features.

DISCUSSION

L-Glutaminase derived from *Achromobacter* 257 can be administered without prohibitive toxicity and rapidly reduces the blood concentration of L-glutamine and L-asparagine to undetectable levels. The enzyme is cytotoxic

in vivo to leukemic lymphoblasts and myeloblasts that are resistant to other drugs; it may also depress normal hematopoiesis, but its toxicity to bone marrow requires assessment in patients without acute leukemia. The pronounced antileukemic activity of this L-glutaminase in man demands further evaluation.

The short half-life of L-glutaminase in the blood may be a disadvantage since frequent injections or iv infusions may be necessary. The *Acinetobacter* L-glutaminase has a half-life of only 1–2 hours in mice but this can be extended to 9–15 hours by succinylation or glycosylation of the molecule (18); these manipulations might similarly affect the *Achromobacter* enzyme. The longer half-life of polymerized L-glutaminase G4 is promising and we are determining the half-life of the more highly polymerized batch G7. A prolonged blood half-life may be unnecessary if tissue depletion of L-glutaminase persists for long periods. In man, we do not know whether tissue depletion is briefer, or more protracted, than blood depletion. Also unknown is the relationship between blood depletion and tissue depletion: do doses in excess of those which produce undetectable levels of L-glutamine in the blood have a greater effect on L-glutamine levels in the tissues? Satisfactory tissue depletion was observed in mice given daily ip L-glutaminase (14), but in these animals the half-life of the enzyme had been prolonged by infection of the mice with lactate dehydrogenase virus. Studies in uninfected mice would be more relevant to the treatment of patients. The optimum schedule for administering L-glutaminase in man might be deduced from the duration of L-glutamine depletion in the blood after single doses, but clinical trials to compare different schedules will be necessary. If effective amino acid depletion can be achieved with a single daily injection, either by the iv, im, or sc route, the therapeutic applicability of L-glutaminase would be enhanced and outpatient treatment would be possible.

The toxicity of *Achromobacter* L-glutaminase is complex and interesting. Effects reported with L-asparaginase (19) and observed by us with this L-glutaminase are: nausea, vomiting, weight loss, somnolence, lethargy, confusion, hypocholesterolemia, mild hypoalbuminemia, and hyperglycemia. The following side effects of L-asparaginase have not, so far, been encountered with *Achromobacter* L-glutaminase: fever, hyperlipidemia, abnormal liver function tests, fatty metamorphosis of the liver, pancreatitis, azotemia, hypofibrinogenemia, and hypersensitivity reactions. Bone marrow depression is recognized with L-asparaginase (19,20) and may occur with L-glutaminase, but our present studies with acute leukemia patients have not adequately demonstrated myelodepression. L-Glutaminase produces marked depletion of L-asparagine in the peripheral blood, and might be expected, therefore, to have all the toxic effects of L-asparaginase, unless simultaneous depletion of L-glutamine protects against some of these effects.

Achromobacter L-glutaminase appears to have some toxic effects which are rare or unknown with L-asparaginase: acidosis, hyperglycemia without ketosis or pancreatitis, hypocalcemia, and hypouricemia. Depletion of L-glutamine might be expected to regularly produce metabolic acidosis, since the mammalian kidney uses L-glutamine as a source of ammonia to excrete excess hydrogen ions. In mice, however, although L-glutaminase produces depletion of L-glutamine in many tissues, the concentration of this amide in the kidney actually rises during therapy, apparently because L-glutamine biosynthesis in renal tissue is increased (14). The metabolic acidosis in patient 9 might have been attributable to the patient's age and possibly reduced renal function; also, he received the largest absolute daily dose of L-glutaminase (40,000 IU) because of his large body surface area. Neither of these factors applied in patient 10, who on two occasions apparently developed a metabolic acidosis overcompensated by respiratory mechanisms, with much reduced plasma bicarbonate and pCO_2. In man there is possibly an enzymatic dimorphism, some individuals possessing a lower capacity to synthesize L-glutamine in the kidney, with resultant enhanced liability to develop acidosis when treated with L-glutaminase. Studies of renal L-glutamine biosynthesis on autopsy material may be the best way to elucidate this point. Close monitoring of acid-base balance is necessary during L-glutaminase administration, until the frequency of metabolic acidosis is established and factors predisposing toward it are identified. Lower doses of L-glutaminase, or the prophylactic administration of alkali, might avert this complication.

Hypouricemia appears to be an interesting but harmless side effect of L-glutaminase. It does not occur in all patients who receive the drug and its mechanism is unknown. Possible factors are an overall mild inhibition of cellular turnover in the normal body tissues, or an enhancement of purine salvage pathways leading to a lesser input of purines into the uric acid-synthetic pathway. The hypocalcemia we have observed has been asymptomatic, but is of interest because of its potential danger, but also its potential use in the management of hypercalcemic states. The conjunction of hypocalcemia and raised serum phosphate levels suggests that L-glutaminase may interfere with the elaboration of parathyroid hormone. The nonketotic hyperglycemia in certain patients produced osmotic diuresis and dehydration in patient 10 and is a serious side effect. No evidence of pancreatitis was present; possibly L-glutaminase interferes with insulin synthesis. Regular monitoring of serum parathyroid hormone and serum insulin levels will be incorporated into future studies with L-glutaminase.

The place of L-glutaminase in leukemia therapy is conjectural. Its activity against L-asparaginase-resistant experimental tumors (12) and in our L-asparaginase-resistant patients suggests its use in patients with ALL and primary or acquired resistance to L-asparaginase. L-Glutam-

inase could prove useful in patients who have developed allergy to both of the available L-asparaginases, and might replace L-asparaginase altogether since it depletes L-glutamine as well as L-asparagine. Simultaneous deprivation of two amino acids, rather than of L-asparagine alone, might confer added therapeutic advantage, although in theory in some cases there could be a protective effect if depletion of L-glutamine nullifies for tumor cells some of the lethal effects of the removal of exogenous L-asparagine. Simultaneous depletion of both L-glutamine and L-asparagine is not encountered during therapy with L-asparaginase from *E. coli* unless excessive doses are used, since the ability of the *Escherichia* enzyme to hydrolyze L-glutamine is only 2%–4% of its activity against L-asparagine (21). L-Glutaminase may have in its own right a place in leukemia treatment, since its spectrum of activity apparently differs from that of L-asparaginase. Its activity in vitro against CLL (9) suggests its study in selected cases of CLL resistant to conventional therapy, and also its use in prolymphocytic leukemia (22), which characteristically is refractory to drugs and irradiation. The activity of L-glutaminase against AML cells in vitro (9) and in patients with AML in the present study suggests that L-glutaminase should now be studied in patients with AML whose disease is at an earlier stage and who are in better general condition. *Achromobacter* L-glutaminase has not as yet undergone clinical trial in tumors other than acute leukemia, and a phase II study in patients with malignant lymphomas and solid tumors refractory to accepted drugs has begun.

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Phase I Study of Succinylated *Acinetobacter* L-Glutaminase-L-Asparaginase [1]

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SUMMARY

Succinylation of *Acinetobacter* L-glutaminase-L-asparaginase (AGA) prolongs the plasma half-life in experimental animals without altering antitumor activity. We designed a protocol to test the human pharmacology, toxicity, and antitumor activity of succinylated AGA. AGA was purified, succinylated, and administered in daily doses of 200–1800 IU/m^2 for 4–30 days to ten children with acute lymphocytic leukemia refractory to all conventional agents and to one patient each with acute myelogenous leukemia, Ewing's sarcoma, ovarian teratocarcinoma, and lymphoepithelioma. Enzyme preparations had an isoelectric point of 5.1–5.7 and a specific activity of 117–150 IU/mg. The plasma half-life was 17–32 hours in four patients. Plasma L-glutamine and L-asparagine were < 2 μM for at least 3 days after a dose of enzyme. Six of eight patients with peripheral lymphoblasts had a 0.4–2.9-log decrease in blast cell count. The patient with acute myelogenous leukemia had a 1.4-log decrease in myeloblast count. A patient with B-cell leukemia had a mixed response and a patient with lymphoepithelioma had a partial response. Toxicity included nausea, vomiting, and anorexia (12 patients), transient fever (seven), myelosuppression (eight), nonketotic hyperglycemia (five), hypoproteinemia (three), and respiratory alkalosis (nine). Myelosuppression was not seen in the two patients with solid tumors and no marrow disease. The plasma ammonia concentration was elevated in all patients but did not correlate with toxicity. A transient acute brain syndrome and coma attributed to the enzyme therapy were observed in one patient. No hepatic or renal toxicity was seen.

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Acinetobacter L-glutaminase-L-asparaginase (AGA) has antitumor activity in L-asparaginase-resistant mouse transplantable tumors, and is cytocidal to human leukemic cells in vitro (1–4). The plasma half-life of the native enzyme is only 1–4 hours in normal mice, rats, and rabbits. Treatment of AGA with succinic anhydride under standard conditions produces a uniform preparation with the same catalytic activity and physical properties as the native enzyme. Furthermore, succinylation markedly increases the plasma half-life in experimental animals (5).

Spiers and Wade have treated patients with acute myelocytic and acute lymphocytic leukemia (ALL) with an *Achromobacter* L-glutaminase-L-asparaginase enzyme (6). The enzyme had a plasma half-life of about 1 hour, necessitating constant infusions of 10,000–20,000 IU/m^2/day to deplete plasma L-glutamine. Patients treated with these infusions had a decrease in blast cell counts.

Succinylated AGA preparations should have a much longer plasma half-life and thereby deplete L-glutamine levels with lower doses. We designed a protocol to test the pharmacology, toxicity, and antitumor activity of succinylated AGA in children with leukemia and other tumors refractory to all conventional chemotherapy. This report describes the phase I plus antitumor activity of the enzyme preparation in the first 14 treated children.

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A preliminary abstract of this work has previously appeared (7).

MATERIALS AND METHODS

Enzyme preparations.—AGA was purified to homogeneity and succinylated in our laboratories by a slight modification of our method (5) suggested by D. J. Salley and J. Roberts (Sloan-Kettering Institute for Cancer Research, Rye, NY). The succinylated enzyme was stored at 5°C as a lyophilized powder; there was no loss of activity during several months of storage. Seven succinylated preparations were administered to the patients; they had specific L-glutaminase activities of 117–150 IU/mg of protein, isoelectric points of 5.1–5.7, and plasma half-lives of 3.9–11.2 hours in normal ICRf male mice (Sprague-Dawley, Madison, Wisc). Endotoxin was monitored by a Limulus amebocyte lysate kit (Worthington Biochemical Corp, Freehold, NJ). The preparations contained 170–690 ng of Food and Drug Administration endotoxin equivalents/1000 IU of L-glutaminase activity. Enzyme activity was assayed in plasma and cerebrospinal fluid by ammonia formation from L-asparagine as previously described (8) except that the ammonia was distilled and assayed by a phenol-hypochlorite reagent (9). The amino acid concentration of sulfosalicylic acid extracts for plasma, urine, and cerebrospinal and pleural fluid was determined on a Joel 5AH analyzer with lithium citrate buffer, pH 2.88, Joel resin AR-50, or Durram resin 1A at 39°C (9).

Determination of antibodies to the enzyme.—Preliminary experiments indicated that ammonium sulfate at 33% saturation precipitated only 10% ± 3% of ^{125}I-labeled AGA alone or mixed with normal rabbit serum or human serum, while 80% ± 5% of ^{125}I-labeled enzyme was precipitated if this ammonium sulfate concentration was added to a mixture of the enzyme and rabbit anti-AGA serum. The specificity of this reaction was tested by adding increasing concentrations of nonlabeled AGA to the iodinated AGA-antibody mixture. The amount of ^{125}I precipitated by antibody decreased linearly as a function of nonlabeled enzyme concentration. Based on these data the following radioimmunoassay was developed. ^{125}I-AGA (0.2 μg) in 50 μl of phosphate buffered saline, pH 7.4, was mixed with 50 μl of patient's serum undiluted or serially diluted with phosphate buffered saline. Controls included ^{125}I-AGA alone, ^{125}I-AGA plus rabbit anti-AGA serum (1:20 and 1:40), and ^{125}I plus normal rabbit serum or human serum. All samples were tested in duplicate. The reagents were incubated at 37°C for 30 minutes and at 4°C for an additional 30 minutes. After the addition of 200 μl of fetal calf serum and 1 ml of 43% saturated ammonium sulfate, the reagents were mixed, incubated at room temperature for 30 minutes, and centrifuged at 1000 × *g* for 15 minutes. The radioactivity in the pellet and supernatant was measured in a gamma spectrometer in order to determine ^{125}I-AGA specifically bound to AGA antibodies.

Immunologic classification of ALL.—All patients with ALL were classified according to the surface characteristics of their bone marrow blast cells. T-ALL lymphoblasts formed E rosettes (10) and reacted with human thymus antisera (11). B-ALL lymphoblasts expressed membrane-intrinsic immunoglobulin of monoclonal nature (12). Common-type ALL lymphoblasts did not possess T or B markers and reacted with heterologous antisera against non-T-, non-B-ALL blast cells (13).

Patients and treatment protocol.—All patients were treated in our Chemotherapy Unit after informed consent was obtained from parents and, when feasible, from the child. The patients had leukemia or solid tumors refractory to conventional chemotherapy. Table 1 lists the pretreatment characteristics. All patients were monitored by daily physical examinations, cbcs, reticulocyte and platelet counts, ammonia, serum electrolytes, pH, glucose, and creatinine. Liver function tests, amylase, uric acid, BUN, and clotting studies were obtained one to two times per week. Weekly calcium and phosphorus levels were followed in seven patients. Timed urine collections were obtained to monitor amino acid and ammonia excretion. Rbcs and platelets were administered, as needed. Insulin levels were kindly determined by Dr. R. K. Kalkoff, Medical College of Wisconsin. The enzyme was dissolved in sterile saline at 90–550 IU/ml and filtered through a disposable 0.22-μ Millex 25-mm filter (Millipore Corp, Bedford, Mass). The enzyme solution was kept at 5°C and used within 3 days or refiltered. All doses were administered iv over 5–10 minutes. On the first day, all patients received a test dose of 25 IU/m^2. A larger dose was administered in 30–60 minutes since no immediate reactions were seen with the test dose. Subsequently, patients were treated with a single daily dose of the enzyme by two methods. The first few patients initially received doses of 200–300 IU/m^2. The dose was gradually escalated every few days. Since most patients tolerated 1000 IU/m^2, in some patients treatment began at this dose level. Two patients received single doses of native AGA. All nonessential medications were stopped. Nausea was treated with iv administration of bolus doses of perphenazine or benzquinamide or with continuous infusions of perphenazine.

RESULTS

Pharmacokinetics

One patient received 525 IU/m^2 of native AGA prior to administration of the succinylated enzyme. The plasma enzyme activity disappeared with a half-life of 26 minutes. One and one-half hours after this dose, the L-glu-

TABLE 1.—Pretreatment summary

Patient No.	Diagnosis	Age (yrs)	Sex	Prior L-asparaginase treatment	Pretreatment blood cell counts × $10^3/\mu l$	
					Wbcs	Blast cells
1	Acute lymphocytic leukemia (common)*	13	M	Yes	16.9	4.3
2	Acute lymphocytic leukemia (common)	5	M	Yes	11.3	7.3
3	Acute lymphocytic leukemia (common)	13	M	Yes	4.1	0.2
4	Acute lymphocytic leukemia (common)	8	F	Yes	1.1	0.5
5	Acute lymphocytic leukemia (common)	12	M	Yes	5.1	0.0
6	Acute lymphocytic leukemia (common)	17	M	Yes	8.3	2.5
7	Acute lymphocytic leukemia (common)	3	M	Yes	269.0	237.0
8	Acute lymphocytic leukemia (T)	14	M	Yes	88.0	54.6
9	Acute lymphocytic leukemia (B) (μλ)	10	M	No	7.4	7.1
10	Acute lymphocytic leukemia (B) (μλ)	11	M	No	12.3	0.0
11	Acute myelogenous leukemia	4	F	No	8.4	1.4
12	Ewing's sarcoma	16	M	No	6.9	0.0
13	Lymphoepithelioma	6	M	Yes	4.5	0.0
14	Ovarian teratocarcinoma	13	F	No	2.8	0.0

*Common, T, and B in parentheses = immunologic subtype as defined in text.

taminase activity was only 0.045 IU/ml of plasma, and L-glutamine was still present in the plasma (8 μM). The L-glutamine level had returned to normal by 3½ hours after the dose. In contrast, after injection of the succinylated enzyme preparations, the plasma enzyme activity disappeared with a half-life of 17–32 hours in the 13 patients tested. The L-glutaminase activity was measured in plasma samples obtained 17–24 hours after each dose. The mean ± SEM level was 0.05 ± 0.02 IU/ml after 200 IU/m^2, 0.13 ± 0.01 IU/ml after 500 IU/m^2, and 0.38 ± 0.02 IU/ml after 1000 IU/m^2. Plasma L-glutamine levels were undetectable (< 2 μM) after doses of 200 IU/m^2 and remained undetectable until the plasma L-glutaminase activity decreased to < 0.06 IU/ml.

During treatment cerebrospinal fluid from four patients had no detectable L-glutaminase activity and normal or elevated levels of L-glutamine (210–1900 μM).

Antitumor Effect

The number of doses, maximum daily dose, and total dose are shown in table 2. The first seven patients listed had ALL with the common immunologic subtype. Six of these patients had lymphoblasts in the peripheral blood. Five of the six patients had a decrease in lymphoblast count of 0.4–2.9 $logs_{10}$. Patient 1 showed an increase in lymphoblast count from 4300 to 36,700/μl during the first 5 days of enzyme therapy of 300–600 IU/m^2/day. The lymphoblast count then fell with continued therapy of 600–1500 IU/m^2/day and reached a nadir of 1900/μl (a 1.3-log decrease from the maximum). Enzyme therapy was stopped at that time because of hyperglycemia, and the lymphoblast count rose to 90,000/μl in 4 days. The best responses were seen in patients 7 and 2. Patient 7 had a rapidly increasing lymphoblast count prior to therapy; this count gradually fell during a month of daily enzyme therapy (fig 1). The lymphoblast count appeared to plateau after several days of treatment at a constant daily dose of enzyme, and then fell again as the dose was increased. The patient had chronic central nervous system leukemia that worsened during this period. He died on March 31, 1977, with signs of brain swelling despite intrathecal cytosine arabinoside and cranial irradiation.

Patient 2 received five daily doses of 1000 IU/m^2 of AGA (fig 2). The enzyme was stopped on August 20, 1978, because of hyperglycemia, but L-glutaminase activity persisted in the plasma, and L-glutamine levels

TABLE 2.—Treatment summary

Patient No.	No. of enzyme doses	Maximum daily dose (IU/m^2)	Total dose (IU/m^2)	Log_{10} change in blast cell count
1	19	1500	14,025	− 0.4
2	5	1000	5030	− 1.5
3	5	1000	5025	− 0.7
4	2	1000	1500	− 1.1
5	6	500	1725	No blast cells
6	12	1300	20,040	+ 0.3
7	29	1800	20,950	− 2.9
8	7	1000	7000	− 0.5
9	2	1000	1555	+ 0.1
10	4*	1200	5070	No blast cells
11	10	1500	10,675	− 1.4
12	10	500	4000	
13	11	1000	11,020	
14	4†	750	3200	

*Includes 1 intrapleural dose of 1800 IU/m^2.
†Includes 1 intrapleural dose of 1200 IU/m^2.

remained undetectable until August 30, 1977. The lymphoblast count fell over this period, and a bone marrow specimen showed necrosis of lymphoblasts and other marrow elements. On August 28, 1977, he began to receive other agents and went into a complete remission.

Patient 8 had T-cell ALL, a large pleural effusion, and mediastinal masses. His lymphoblast count increased from 55,000 to 100,000/μl on the first day of therapy but subsequently fell to 16,500/μl. The pleural fluid lymphoblasts and mediastinal masses were not affected clinically by treatment.

Patient 9 with B-cell ALL could not be evaluated because of early death. The other patient with B-cell ALL had no circulating lymphoblasts but had large loculated pleural effusions, a right testicular mass, and a subcutaneous nodule. He received three daily iv doses of 1000–1200 IU/m^2 and an intrapleural dose of 1700 IU/m^2 on Day 4. The size of the subcutaneous nodule and the testicular mass decreased by > 50%. Three pleural taps were performed. The first, after three iv doses, and the second, 2 days after the intrapleural dose, showed no tumor cells and no L-glutamine or L-asparagine in the fluid. The third tap, 2 days later, showed many viable tumor cells and low levels of L-glutamine (3 μM).

The patient with acute myelogenous leukemia had a decrease in blast cell count of 1.4 logs, but therapy was stopped because of nausea, vomiting, and diarrhea.

Patient 13 had a lymphoepithelioma of the left parotid area. The tumor was responsive to 6-mercaptopurine and methotrexate but not to prednisone, L-asparaginase, Adriamycin, or cyclophosphamide. The jaw lesions increased rapidly in the week prior to enzyme therapy. During L-glutaminase therapy the lesions softened and their volume decreased about 50%. In the week following cessation of the enzyme therapy the lesions again rapidly increased in size.

The patients with Ewing's sarcoma and ovarian teratocarcinoma showed no response to the enzyme therapy.

Toxicity

Patient 9 died of intestinal obstruction 1 day after the second dose of enzyme. The toxicity seen in the other patients is summarized in table 3. Nausea, vomiting, and anorexia began about 1 hour after the first dose and lasted throughout the treatment period. Diarrhea developed in two patients. These symptoms were controlled with drugs in most patients. Therapy was discontinued in two patients because of gastrointestinal toxicity. Eleven patients had a weight loss of 0.3%–1.4% of their body weight

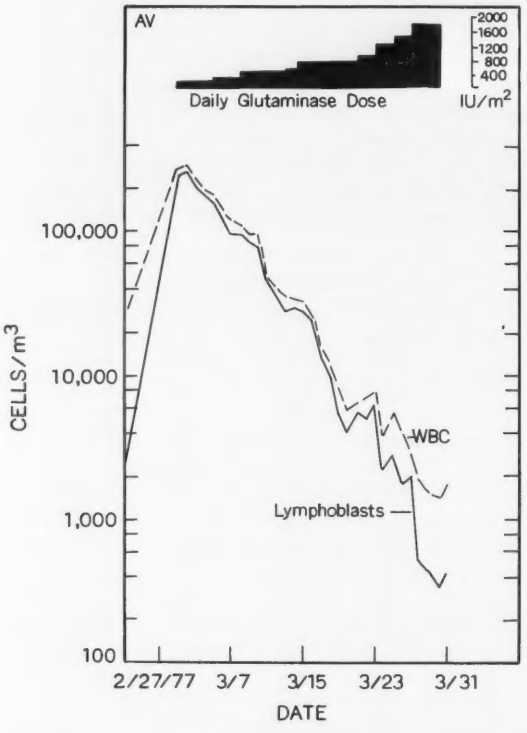

FIGURE 1.—Effect of enzyme therapy on wbc and lymphoblast counts in patient 7.

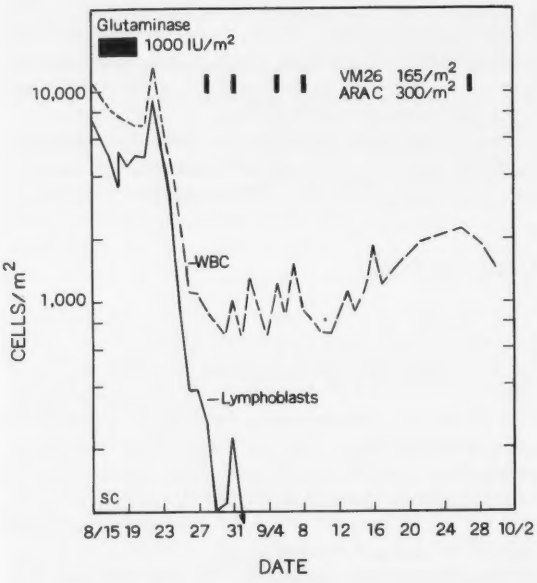

FIGURE 2.—Effect of enzyme therapy on wbc and lymphoblast counts in patient 2. Doses of L-glutaminase and other drugs are indicated at top of fig; ARAC = cytosine arabinoside.

TABLE 3.—Toxicity of succinylated AGA

Toxic effects	No. of patients with toxic effects/ No. evaluated
Nausea, vomiting, and anorexia	12/13
Transient fever	7/13
Glucose > 160 mg/dl	5/13
Albumin < 2.8 g/dl	3/13
Fibrinogen < 160 mg/dl	3/10
pH > 7.48	9/13
Total CO_2 < 20 mEq/liter	12/13
Hemoglobin < 10 g/dl	8/13
Reticulocytes < 0.5%	8/13
Granulocytes < 1000/μl	5/13
Lymphocytes < 500/μl	8/13
Platelets < 50,000/μl	8/13

per day. A transient fever of 1°C–2°C developed about 1 hour after the first dose in six patients and after the first two doses in one patient. This fever might have been caused by the small amounts of endotoxin in the preparations.

Five patients developed hyperglycemia without ketosis or acidosis after five to 19 doses of 500–1500 IU/m²/day. Peak blood sugar levels were 170–590 mg/dl. Blood sugar levels became normal within a few days after enzyme therapy was stopped in four patients (two patients received 3 and 10 units of insulin). Blood sugar levels fell toward normal with insulin therapy in the fifth patient but he died of a gastrointestinal hemorrhage. Insulin levels were < 30 micro-units/ml during the hyperglycemia in four of these patients.

Other laboratory tests showed low albumin and fibrinogen in three patients, prolongation of prothrombin time by 2–4.5 seconds in eight patients, and a partial thromboplastin time of > 39 seconds in two patients. Nine patients developed a mild respiratory alkalosis with venous total CO_2 levels of 14–20 mEq/liter. Serum calcium was 7.8 and 8.5 mg/dl in two of eight patients tested. No patients had elevated serum amylase, SGOT, BUN, creatinine, or bilirubin. Urinalyses were all normal.

Four patients with prior central nervous system leukemia became comatose during therapy. Patients 5 and 7 had chronic meningeal leukemia that progressed during enzyme therapy. Patient 6 had hyperglycemia and a severe gastrointestinal hemorrhage. These patients died. Patient 4 became unresponsive with intermittent screaming and decorticate posturing 7 hours after the second dose of 1000 IU/m²/day. Left-sided weakness and generalized seizures developed but there was no papilledema or respiratory difficulty. The cerebrospinal fluid showed no cells. An electroencephalogram showed diffuse slowing. One dose of mannitol was given and dexamethasone was begun. The patient awakened in 5 days, at the same time that L-glutamine reappeared in the plasma. All neurologic signs were normal in 7 days.

Plasma ammonia levels were elevated in all patients during therapy; maximal values were 320–1130 μg/dl. The plasma ammonia did not correlate with the level of consciousness.

Myelosuppression was seen in eight patients. All these patients had bone marrow replacement by leukemia or Ewing's sarcoma, extensive prior chemotherapy, and bone marrow irradiation (patient 12). Two patients without bone marrow disease (Nos. 13 and 14) had no myelosuppression.

No anaphylactic reactions occurred. Plasma was tested for antibodies to AGA during and up to 11 days after cessation of enzyme therapy. No antibodies were detected. Furthermore, the rate of disappearance of plasma enzyme activity did not increase during enzyme therapy.

Autopsies were performed on patients 1, 2, 6, 9, 10, and 14 who died 19, 200+, 3, 1, 17, and 48 days, respectively, after the last dose of enzyme. In patient 10 focal areas of pancreatic necrosis and slight proximal renal tubular swelling were observed, and in patients 6 and 10 fatty infiltration of the liver was observed. Other pathologic findings were consistent with widespread malignancy.

DISCUSSION

This preliminary study has shown that a simple chemical modification of AGA produces a preparation with a long half-life in patients. The succinylated enzyme can deplete circulating levels of L-glutamine and L-asparagine with one hundredth the daily doses required with the native enzyme used by Spiers and Wade (6). Furthermore, L-glutamine remains depleted when the succinylated enzyme is administered as a single daily dose or even less frequently.

Several types of severe toxicity were encountered. The coma seen in three of four patients with prior meningeal leukemia could be attributed to other causes. In one patient the coma appeared to be related to the L-glutamine and L-asparagine depletion. Cranial irradiation, prior systemic and intrathecal chemotherapy, or chronic meningeal leukemia could predispose these patients to some adverse effect of the enzyme therapy. Similar neurologic dysfunction has been seen with L-asparaginase (14–17). As noted with L-asparaginase, ammonia levels did not correlate with this toxicity (16).

Hyperglycemia with low insulin levels has been reported with *Escherichia coli* L-asparaginase, but the frequency seems to be higher with succinylated AGA (15–17). The low insulin, hypoalbuminemia, hypofibrinogenemia, and prolonged prothrombin time seen in a few patients suggest that the enzyme may be inhibiting the synthesis of several important proteins. The enzyme was stopped when hyperglycemia appeared. In the future we plan to maintain the patients with insulin and to continue therapy.

Myelosuppression is difficult to evaluate in patients with extensive bone marrow disease. Two patients in this study had solid tumors, no marrow involvement, and no myelosuppression. AGA has been shown to produce myelosuppression in mice (18). Therefore, further studies are needed to evaluate this toxicity.

The acute respiratory alkalosis is unexplained but could be due to hyperventilation associated with nausea and vomiting.

During treatment, the level of L-glutamine in each tissue and fluid space will depend on the extracellular levels of L-glutaminase, the rate of transport of L-glutamine out of the tissue or cells lining the space, and the rates of syntheses and utilization of L-glutamine. This critical balance is illustrated by the difference in the antitumor activity in the various loculated pleural effusions in patient 10. The two fluid samples with no detectable L-glutamine had no viable tumor cells, while the sample that contained L-glutamine had numerous tumor cells. This balance is also shown by the observations that cerebrospinal fluid L-glutamine was not depleted by systemic AGA therapy and that meningeal leukemia worsened during a dramatic antileukemic effect in the blood (patient 7).

These studies show that succinylated AGA has antitumor effect in ALL, acute myelogenous leukemia, and, possibly, lymphomas. These results confirm previous in vitro (4) and patient (6) studies. Further studies are needed to determine the maximum-tolerated dose, best dose schedule, spectrum of antitumor activity, and potential usefulness of AGA in the treatment of patients.

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Antitumor Effects of Azaserine and DON[1]

Joseph H. Burchenal[2,*]

The antitumor activity of the two closely related antibiotics, azaserine and 6-diazo-5-oxo-norleucine (DON), was discovered by the Parke Davis group (1,2) who were working in conjunction with Stock et al and Clarke et al at the Sloan-Kettering Institute (3,4). Preclinical pharmacologic studies were done by Sternberg et al (5,6), and the activity against the chick and rat embryo was determined by Murphy et al and Dagg et al (7,8). Both compounds were given phase I trials and, since they were both active orally, most of the phase II studies were done by the oral route with the drug given daily (9,10). Tarnowski and Stock (11) have demonstrated the increased activity of these compounds in combination with 6-mercaptopurine, and, for that reason, they were tried in combination with 6-mercaptopurine in the treatment of children with acute leukemia. Preliminary studies suggested a better remission rate and longer remission duration with a combination of azaserine and 6-mercaptopurine each given at a dose of 2.5 mg/kg daily orally than had previously been seen with 6-mercaptopurine alone (12), but a randomized clinical trial by the Children's Cancer Study Group A showed no significant difference. A few patients treated with this combination, however, have remained free of disease for 23+ years from the onset of treatment. Clinical studies of DON reported by Magill et al (10) in a wide variety of solid tumor patients (with DON given at doses pushed to the limiting oral toxicity) showed responses in two of 14 patients with breast cancer, two of nine with lung cancer, one of six with colon cancer, one of five with uterine cancer, and one of four with lymphoma. Most of these patients were treated on a daily basis, with doses of 0.2–0.4 mg/kg daily for 7–14 days producing oral toxicity despite the fact that animal data had shown better effects when the drug was given every second day than when it was given every day and that much larger doses could be given without serious toxicity. Only one patient was treated at less than daily intervals. This patient tolerated considerably more drug (1.6 mg/kg every fourth day) with toxicity limited to nausea and vomiting and no sore mouth or diarrhea.

TABLE 1.—Effect of schedule on antileukemic effect of DON on L1210/0

Dose (mg/kg)	Schedule*	ILS† (%)
45	q4d × 3	76
30	q4d × 3	124
20	q4d × 3	117.3
13.3	q4d × 3	104
9.0	q2d × 5	84
6.0	q2d × 5	77.3
4.0	q2d × 5	108
2.7	q2d × 5	96
0.9	qd × 9	15
0.6	qd × 9	40
0.4	qd × 9	46.7
0.27	qd × 9	73.3
0.18	qd × 9	57.3

*Treatment started 24 hrs after inoculation of 10^6 leukemic cells.
†ILS = increase in lifespan.

Somewhat later, Karnofsky et al (13) reported three remissions in patients with chorioadenoma destruens but felt that DON was not as active as actinomycin D or methotrexate and that it should not be used in choriocarcinoma. As yet, no concerted attempt to use the drug on an every-4-day or once weekly schedule has been made.

Experiments in our laboratories using mouse L1210 leukemia and P388 leukemia show marked differences in the optimal amount of DON when given daily (0.27 mg/kg), every other day (4 mg/kg), or every fourth day (30 mg/kg). In these experiments (table 1) it appeared that the every-2-day or every-4-day schedule produced better therapeutic results than the daily dose schedule. Preliminary studies on combination therapy for mouse L1210 leukemia showed additive to synergistic effects with 6-thioguanine, *cis*-dichlorodiammineplatinum(II), VP-16-213, and Adriamycin (table 2) when the drugs were given on Days 3, 7, 11, and 15.

Based on these data, it is suggested that when DON goes into clinical trial again it should be given iv on an every-4-day or a once weekly dose schedule, and it should be given adequate trial in phase II studies in solid tumors, particularly carcinoma of the lung, uterus, colon, and breast.

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TABLE 2.—Results of study to determine best drug combinations with DON in delayed L1210/0

Drug	Dose (mg/kg) on Days 3, 7, 11, and 15	Survival (days)	ILS* (%)
Control		9.2	—
DON	16	15.7	70
DON	8.0	14.2	54
DON	4.0	11.7	27
DON	8.0	23.4	154
VP-16-213	20	—	
DON	8.0	19.7	114
cis-Dichlorodiammineplatinum(II)	3.0	—	
DON	8.0	17.6	91
Adriamycin	3.0	—	
DON	8.0	20.2	119
6-Thioguanine	1.0	—	
cis-Dichlorodiammineplatinum(II)	3.0	12.7	38
6-Thioguanine	1.0	9.7	5
Adriamycin	3.0	11.2	22
VP-16-213	20	19.7	114

*ILS = increase in lifespan.

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Azaserine, DON, and Azotomycin: Three Diazo Analogs of L-Glutamine With Clinical Antitumor Activity

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SUMMARY

A review of the clinical data on azaserine, DON, and azotomycin reveals that these agents have limited but definite antitumor activity. All three drugs are analogs of L-glutamine and contain a diazo group. They have been studied as single agents in a wide variety of human malignancies and have also been included in trials using combination chemotherapy. Most of these studies were performed early in the history of clinical trials and, therefore, the method of reporting results and the evaluation criteria were quite different from those in use today. A renewed interest in these agents has been triggered by the remarkable activity of DON and azotomycin against human tumor lines implanted into nude mice. On the basis of this activity and the clinical data we have compiled, we feel that new clinical trials with these agents are warranted.

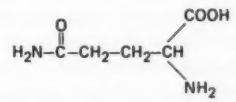
[Cancer Treat Rep 63:1033–1038, 1979]

Azaserine, DON, and azotomycin are antibiotics produced by *Streptomyces* species. They are diazo analogs of L-glutamine (figs 1 and 2). All three agents interfere with several reactions in which L-glutamine participates and specifically they inhibit de novo purine biosynthesis by interfering with transamidation reactions requiring L-glutamine as the nitrogen-group donor (1,2). We will present a review of the clinical data available for these three drugs, together with new data on their activity in experimental tumor models.

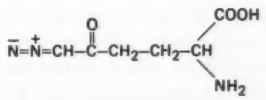
Azaserine

Azaserine is one of the earliest antineoplastic agents registered with the Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute. Its chemistry and biologic activity have been extensively reviewed (2,3). The mechanism of action has been shown to be the inhibition of various L-glutamine amidotransferases, but in high concentration it has alkylating properties (3,4); these are probably not important in vivo since only the L-isomer is active as a chemotherapeutic agent. Recently, a re-

L-GLUTAMINE



DON: 6-diazo-5-oxo-L-norleucine



AZASERINE

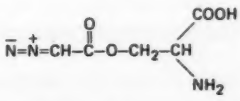


FIGURE 1.—Structures of DON and azaserine compared to L-glutamine.

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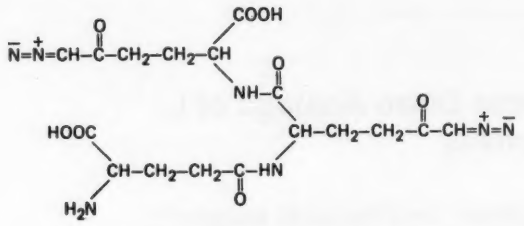

FIGURE 2.—Structure of azotomycin.

markable synergism between azaserine and other antimetabolites was demonstrated in an animal tumor model (5) and in cell culture (6). Compared to other L-glutamine antagonists, such as DON, azaserine produces greater pancreatic and hepatic toxicity in animals, but, as an oncolytic drug, it is somewhat less potent on a molar basis. This discrepancy probably reflects the differing pharmacologies of these agents.

The first clinical trial of azaserine was reported by Ellison et al (7) who used a crude filtrate of the *Streptomyces* broth before the structure of the compound was known. This culture broth inhibited the growth of mouse sarcoma 180. Concomitant with the performance of this trial, the active principle in the broth was isolated, identified as azaserine, and later synthesized. In man, pure azaserine was tolerated up to a dose of 8–10 mg/kg/day for 1 or 2 weeks. The toxic effects included oral mucositis, nausea, vomiting, mild leukopenia, and, at times, jaundice. The therapeutic results were limited to minimal objective responses in eight patients; all of the responses were of brief duration and none was complete (table 1). Azaserine was ineffective in a prospective randomized trial against multiple myeloma (8), where there were no objective responses among nine patients.

The Central Oncology Group recently conducted a phase I study of the iv administration of azaserine in several dose schedules (Wilson WL, personal communication). The results indicate that doses as high as 30 mg/kg are tolerated when given iv twice weekly for 4 weeks; minimal toxicity was seen at a dose of 16 mg/kg/day × 5 iv. When the drug was infused over 4–5 hours daily for 5 days, the moderate toxicity included hepatic toxicity, bone marrow suppression, nausea, and vomiting. The recommended safe dose, which causes mild toxicity, was 7.5 mg/kg by a 4–5-hour infusion daily for 5 days. There were few responses in this study (table 1), but the results may not reflect the true response rate for azaserine since most of the patients did not receive the optimal dose.

TABLE 1.—Single-agent activity of azaserine

Disease	No. of responders/ No. of evaluable patients	Ref No.
Acute leukemia	3/18	7
Hodgkin's disease	5/19	7
Myeloma	0/9	8
Lymphoma	2/3	COG*
Head and neck carcinoma	1/6	COG
Colorectal adenocarcinoma	1/20	COG
Breast carcinoma	0/7	COG
Miscellaneous solid tumors	0/14	7
	0/25	COG

*Central Oncology Group (Wilson WL, personal communication).

Azaserine in Combination Chemotherapy

As shown in table 2, two of 15 multiple myeloma patients who failed to respond to or had relapsed after previous chemotherapy responded to a combination of azaserine and 6-thioguanine (9). This combination was considered ineffective in the terminal phase of chronic myelocytic leukemia, since only two of 22 patients achieved brief partial responses (10). However, azaserine and 6-thioguanine were moderately effective in solid tumors, especially in breast cancer, but the toxicity was severe (11).

Burchenal et al (12) showed somewhat promising results with the combination of azaserine and 6-mercaptopurine (6-MP) in a pilot study of childhood acute leukemia. A cooperative study group (Leukemia Chemotherapy Cooperative Study Group A) followed this lead, but failed to confirm any advantage for this combination over 6-MP alone (13). However, Hall et al (14) reported that two patients with 6-MP-resistant leukemia subsequently achieved remission when 6-MP was combined with azaserine. Leventhal et al (15) conducted a study comparing azaserine and L-asparaginase to L-asparaginase alone in acute lymphocytic leukemia. The response rate for the combination was impressive (11 responses among 16 patients), but the responses were of short duration and the response rate did not differ significantly from that produced in the group receiving L-asparaginase alone. In this study, azaserine was given orally and an additional study was designed using iv azaserine with a higher dose of L-asparaginase (16). However, the latter study again failed to produce a better response than L-asparaginase alone.

DON

DON is also an early anticancer agent and its chemistry, pharmacology, toxicology, and experimental antitumor activity have been extensively reviewed (1,3,17,18). Table 3 summarizes the single-agent activity of DON against various tumors. Magill et al (19) conducted a phase I evaluation of several schedules of DON; most of

TABLE 2.—Activity of azaserine in combination chemotherapy

Azaserine and—	Disease	No. of responders/ No. of evaluable patients	Ref No.
6-Thioguanine	Myeloma	2/15	9
6-Thioguanine	Terminal phase of chronic myelocytic leukemia	2/22	10
6-Thioguanine	Breast carcinoma	4/7	11
	Head and neck carcinoma	1/2	11
	Other solid tumors	0/7	11
6-MP	Childhood acute leukemia	25/39	12
		43/58	13
L-Asparaginase	Acute lymphatic leukemia	11/16	15
		8/14*	16

*Iv azaserine.

TABLE 3.—Single-agent activity of DON

Disease	No. of responders/ No. of evaluable patients	Ref No.
Breast adenocarcinoma	2/14	19
Lung carcinoma	2/9	19
	0/18	20
Gastric and colon carcinomas	1/6	19
Melanoma	0/4	19
	0/5	20
Lymphoma	1/4	19
	0/7	20
Leukemia	0/1	19
Hodgkin's disease	2/11	20
Choriocarcinoma	3/4	21
Miscellaneous tumors	1/9	19

the patients received daily administration of the drug either orally, im, or iv. The dose recommended for this schedule was between 0.1 and 0.3 mg/kg/day orally or parenterally. The toxicity included mucositis, diarrhea, nausea, vomiting, and bone marrow suppression. It was noted that intermittent administration, every 4 days, greatly reduced the toxicity.

The Veterans' Administration Cancer Chemotherapy Study Group compared the effectiveness of DON and mechlorethamine (20) in patients with bronchogenic carcinoma, Hodgkin's disease, lymphosarcoma, and melanoma (table 3). The drug was given orally at a dose of 0.2 mg/kg/day for 30 days. Very few patients responded to DON; mechlorethamine was more effective in all of the diseases investigated, except in melanoma where neither drug was effective. DON was quite effective in four patients with choriocarcinoma, achieving an apparent cure in two patients and an additional transient complete response in a third (21). Methotrexate, which was more effective and less toxic, soon replaced DON in the treatment of choriocarcinoma.

Combined with other chemotherapeutic agents (table 4) DON has been effective in testicular cancer, with three of four patients responding and one patient apparently cured (22). Sixty-three of 94 children with acute leukemia treated with the combination of DON and 6-MP achieved complete remission in a study by Sullivan et al (23). This randomized study showed a slight advantage for the combination over 6-MP alone, but this was balanced by a marked increase in toxicity, especially mucositis and other gastrointestinal symptoms.

DON has been effective in correcting hypercalcemia and hypercalciuria resulting from lytic bone metastases (mainly from breast cancer). As a single agent, DON significantly improved these conditions in four of seven patients, and four of four patients responded to the administration of DON with 6-MP (24).

Azotomycin

Azotomycin, a polymer of two molecules of DON and an L-glutamic acid residue (fig 2), probably becomes active in vivo by conversion to DON (25,26). A phase I study of azotomycin revealed that its toxic effects consisted of leukopenia, thrombocytopenia, nausea, and vomiting (27). The dose recommended for phase II trials was 5 mg/kg/day × 5, to be repeated after 1 month. However, subsequent studies used a 10-day schedule with a dose of 2 mg/kg/day.

TABLE 4.—Activity of DON in combination chemotherapy

DON and—	Disease	No. of responders/ No. of evaluable patients	Ref No.
Miscellaneous alkylating agents	Testicular carcinoma	3/4	22
6-MP	Acute childhood leukemia	63/94	23

TABLE 5.—Single-agent activity of azotomycin

Disease	No. of responders/ No. of evaluable patients	Ref No.
Colorectal carcinoma	11/64	28
	0/2	29
Sarcoma	4/14	28
	0/1	29
	1/13	30
Lung cancer	2/21	28
	0/2	29
Melanoma	3/21	28
	0/1	29
Lymphoma	2/7	28
Breast carcinoma	1/15	28
Head and neck squamous cell carcinoma	1/21	28
	0/1	29
Miscellaneous solid tumors	1/32	21
	0/2	29

The single-agent activity of azotomycin is summarized in table 5. The Central Clinical Drug Evaluation Program conducted a broad phase II study (28) which showed promising results in colorectal cancer, sarcoma, and melanoma. Hill et al (29) did not see any responses in a small group of patients with a variety of tumors. More recently, Chang and Wiernik (30) failed to confirm the single-agent activity in sarcoma patients, all of whom had received prior treatment.

Azotomycin combined with 5-fluorouracil (table 6) showed a 45% response rate in colon cancer (31), but only one partial response occurred among five leukemic patients treated with combined azotomycin and L-asparaginase (16).

Activity of Azotomycin and DON on Human Tumor Xenografts

Xenografts of human tumors implanted in nude mice may predict tumor response to anticancer agents, hopefully with more specificity than that shown by previously used transplantable animal tumor screens (32,33).

Since azotomycin has shown a promising degree of activity in colorectal cancer, it was decided to evaluate its effect in a panel of human tumor xenografts transplanted into nude mice. The therapeutic effects of azotomycin were compared to those of other more conventional chemotherapeutic agents and to results with DON, which is probably the active metabolite of azotomycin (26). Four human tumor lines were available for testing, ie, two colon adenocarcinomas (CX-1 and CX-2), one tumor line derived from a patient with small cell lung cancer (LX-1), and one tumor line from tumor tissue acquired from a premenopausal breast cancer patient (MX-1) (34).

The activity of azotomycin, DON, and six other anticancer drugs against these human tumor xenografts is summarized in table 7.

DON and azotomycin showed marked effectiveness in the lung cancer xenograft, whereas none of the conventional agents tested were active. In the colon cancer xenograft CX-2, DON was the most active, while azotomycin, 5-fluorouracil, and cyclophosphamide were only minimally effective. The other colon xenograft (CX-1) was resistant to all three drugs. The breast tumor xenograft was sensitive to several agents but especially to DON, azotomycin, and cyclophosphamide.

Conclusions

A review of the clinical trials with azaserine, DON, and azotomycin shows that only limited clinical benefit has been encountered. However, it should be remembered that a large proportion of the patients received daily oral treatment, which is perhaps not optimal.[4] In addition, the studies span several decades and many were only phase I trials using variable drug preparations in which most of the patients received less than the optimal dose. Nevertheless, definite antitumor activity was repeatedly established. In fact, the activity of azotomycin in colon cancer is particularly noteworthy. Therefore, we

[4]Support for this contention is provided by an inspection of the chemistry of this whole family of drugs. The diazo function is known to be labile at the pH of the gastric contents.

TABLE 6.—Activity of azotomycin in combination chemotherapy

Azotomycin and—	Disease	No. of responders/ No. of evaluable patients	Ref No.
5-Fluorouracil	Colon cancer	10/22	31
L-Asparaginase	Acute lymphatic leukemia	1/5	16

TABLE 7.—Drug activity against human tumor xenografts*

Drug	Activity†			
	Lung (LX-1)	Colon (CX-2)	Colon (CX-1)	Breast (MX-1)
DON	+ +	+ +	–	+ +
Azotomycin	+ +	+	–	+ +
Methotrexate		–		+
6-MP	–	–		–
5-Fluorouracil		+	–	+
Cyclophosphamide	–	+		+ +
Adriamycin	–			
BCNU		–		+

*Modified from refs 34 and 35.

† + (retards tumor growth), + + (elicits tumor regression), – (not active).

may hope that the effectiveness of azotomycin and DON in the human colon cancer xenografts, and in other tumors implanted into nude mice, will predict for significant therapeutic value against these malignant tumors in man.

Accordingly, the National Cancer Institute has planned new studies using unexplored schedules and optimal doses of these agents, and DON will soon become available for renewed clinical studies.

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Possibilities for a Role of L-Glutamine and L-Asparagine Antagonists in the Treatment of Gastrointestinal Cancer

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Although experience with L-asparagine and L-glutamine antagonists in gastrointestinal cancer is limited, there is a body of evidence to suggest a role for these agents in this group of diseases. In gastric cancer, however, there appears to be no important experience with L-glutamine or L-asparagine antagonists. The comprehensive review by Comis and Carter (1) of drug therapy in gastric cancer made no reference to agents of this class.

In colon cancer there is evidence that the L-glutamine antagonist, azotomycin, may be of clinical value. In 1968, a report from the Central Clinical Drug Evaluation Program (2) cited a trial of azotomycin at a dose of 2 mg/kg/day × 10 days in patients with advanced measurable colon cancer. There was a 17% response rate (11 responses among 64 patients). Subsequently, Weiss et al (3) reported ten responses among 22 patients with a combined regimen of azotomycin and 5-fluorouracil (5-FU). In this case, the initial dose was 5 mg/kg/day of 5-FU and 1.0 mg/kg/day of azotomycin for 3 days, given by rapid iv injection. After 3 days' rest, the patient was given 5 mg/kg of 5-FU and 1.0 mg/kg of azotomycin every other day until toxicity developed. These results have not been adequately followed up although the data suggest a role for these agents in colon cancer therapy.

In the Central Clinical Drug Evaluation Program study (2), there was also a report of one complete response among four patients with pancreatic cancer. Such observations need to be pursued. Lessner et al[2] have concluded a pilot study of L-asparaginase in pancreatic cancer. The decision to conduct the trial was based on experiments in tissue culture, in which L-asparaginase was tested against cell lines derived from various human tumors, including some pancreatic cell lines. Cell lysis by L-asparaginase was found to occur preferentially in the pancreatic tumor lines. Accordingly, ten patients were treated with L-asparaginase in 10-day courses. Initially, the dose was 1000 units/kg/day × 10 days, but because of toxicity in the form of hypotension, the dose was reduced to 1000 units/kg/day × 2 days followed by 500 units/kg/day × 8 days. No clinical responses were seen in these ten patients.

This trial suggests a role for tissue culture methods in evaluation of amino acid depletion in clinical therapy. There are several approaches to this problem, one of which is that proposed by Woolley et al (4). These investigators examined the sensitivity of L1210 cells in culture to the depletion of the single amino acid, L-tryptophan. The methods used included examination of cell proliferation and viability, incorporation of radioactive precursors into histones, non-histones, whole-cell protein, and DNA, and cell microfluorometry. It was found that after depletion of L-tryptophan, the cells proliferated slowly and remained viable for the first 24 hours, after which progressive cell death occurred. Whole-cell protein and non-histone protein syntheses were less sensitive to L-tryptophan deprivation than were DNA and histone syntheses, so that DNA synthesis declined more rapidly than protein synthesis. Cell microfluorometric analysis showed that, after L-tryptophan deprivation, there was an initial accumulation of cells in S and G_2, followed by an arrest in G_1.

Subsequent studies in our laboratory, presently in progress, have examined the effects of L-asparagine and L-glutamine deprivation on the L1210 line. The results indicate that deprivation of L-asparagine produces immediate reduction in DNA and protein synthesis to low levels (about 10% of control), but with a maintenance of cell viability beyond that seen with L-tryptophan deprivation. Complete deprivation of L-glutamine, by contrast, produces a very lethal deficit to the cells, and cell proliferation is quite sensitive to even partial depletion of this amino acid. Partial depletion of L-glutamine is synergistic with L-asparagine deprivation in this system, in terms of terminating cell DNA synthesis and viability. On the basis of these experiments we conclude that L-glutamine > L-tryptophan > L-asparagine with regard to the lethality of the single amino acid depletion in L1210 cells.

These results indicate that such methods can be applicable to the screening of other cell lines for the effects of depletion of these and other amino acids. These may allow the identification of particularly lethal amino acid

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[2]Lessner HE, Kaplan R, Valenstein S, et al. Treatment of pancreatic carcinoma with L-asparaginase. Presented at the International Conference on Gastrointestinal Cancer, Tel Aviv, Israel, Nov 8, 1977.

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deficiencies that can be exploited clinically in gastrointestinal cancer and other tumors.

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Changes in Amino Acid Metabolism Caused by 6-Azauridine Triacetate: Relevance to Cancer Treatment

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SUMMARY

6-Azauridine triacetate (6-AzUrd-TA) administration causes changes in amino acid metabolism both in experimental animals and in man. This effect is dose-related. Amino acid changes caused by 6-AzUrd-TA resemble those in inborn homocystinuria, β-alaninemia, and hyperhistidinemia. Inhibition of certain enzymes using pyridoxal phosphate as a coenzyme appears to be the common denominator for these changes. There is supportive evidence suggesting that homocystinemia and thrombotic episodes, both caused by 6-AzUrd-TA, are related. These results also reveal that anticancer drugs other than amino acid analogs and amino acid-depleting enzymes may cause significant changes in amino acid metabolism. Their detection and correlation with the therapeutic or adverse effects can be used as an alternative method for studying the relevance of amino acid changes to the treatment of cancer.

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Although this symposium primarily focused on amino acid analogs and amino acid-depleting enzymes, this paper will present results which reveal that other anticancer drugs may also cause significant changes in amino acid metabolism. Our studies with 6-azauridine triacetate (6-AzUrd-TA) are used as an example, and their relevance to cancer treatment is discussed.

6-AzUrd-TA is a pyrimidine nucleoside analog, developed both in Czechoslovakia (1,2) and in the US (3). It revealed beneficial effects in the treatment of psoriasis (4–7), mycosis fungoides (8,9), trophoblastic malignancies (10,11), erythroleukemia (12), and polycythemia vera (13). This drug is commercially available in Europe and was marketed in the US for treatment of psoriasis until it was recalled in August 1976 by the Food and Drug Administration because of the life-threatening thromboembolic episodes reported in about 4% of treated patients (7,14).

The mode of action of 6-AzUrd-TA has been elucidated as inhibition of pyrimidine de novo synthesis at the orotidine-5′-phosphate decarboxylation step (3). 6-AzUrd-TA is converted to 6-azauridine-5′-phosphate, which is the specific inhibitor of orotidine-5′-phosphate decarboxylase (15). Consequently, the precursors of pyrimidine de novo synthesis, including carbamyl-L-aspartic acid (16), orotic acid, and orotidine, are formed in increased amounts and excreted in the urine (16).

Our interest in studies on amino acid metabolism after 6-AzUrd-TA administration was generated by both biochemical as well as clinical considerations.

(*a*) Carbamyl-L-aspartic acid, formed in increased amounts after 6-AzUrd-TA administration, could be cleaved by carbamyl-L-aspartate decarboxylase to yield carbamyl-β-alanine, which is further catabolized to β-alanine (17). β-Alanine is also a degradative product of uracil, formed from uridine phosphate. Patients treated with 6-azauridine have, in some instances, an increased production of uridine-5-phosphate (16), in spite of the orotidylic acid decarboxylate inhibition. This surprising biochemical effect has not yet been fully elucidated.

(*b*) Increased urinary excretion of taurine was reported after ultraviolet irradiation or administration of corticosteroids in psoriatic patients (18), and beneficial effects were also achieved by a diet low in this amino acid (19,20).

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TABLE 1.—Serum amino acid changes after 6-AzUrd-TA*

Ref No.	Subject	Dose	Amino acid levels					
			L-Methionine		L-Homocystine		β-Alanine	
			Before	After	Before	After	Before	After
25	Rabbits	1 g/kg/day	37.8 ± 2.4	45.8 ± 4.3	†	28.0 ± 8.0	†	63.9 ± 20.6
26	Man (progressive systemic sclerosis)	9 g/day	20.4 ± 2.7	67.3 ± 14.2	†	42.3 ± 14.3	†	23.7 ± 3.6

Ref No.	Subject	Dose	Amino acid levels					
			β-AIBA‡		L-Threonine		L-Histidine	
			Before	After	Before	After	Before	After
25	Rabbits	1 g/kg/day	†	21.8 ± 8.4	NM§	NM§	NM§	NM§
26	Man (progressive systemic sclerosis)	9 g/day	NM§	NM§	121.6 ± 11.8	196.2 ± 26.3	67.9 ± 6.7	120.7 ± 14.7

*Results are expressed in nmols/ml of serum and represent the mean ± SEM of 1 determination in each of 5 animals or in each of 7 patients.
†Not detectable, ie, < 2 nmols/ml.
‡β-Aminoisobutyric acid.
§Not measured.

TABLE 2.—Urine amino acid levels after 6-AzUrd-TA*

Ref Nos.	Disease	Dose	Amino acid levels					
			L-Homocystine†		β-Alanine		β-AIBA‡	
			Before	After	Before	After	Before	After
21	Psoriasis	400 mg/kg/day	§,¶	0.26–3.4	§	0.9–1.29	NM**	NM**
23,24	Mycosis fungoides	250 mg/kg/day	§	0.14	§	0.41–0.6	§	0.15
23,24	Rheumatoid arthritis	50 mg/kg/day	§	¶	§	0.07–0.15	§	0.03–0.22
26	Progressive systemic sclerosis	9–12 g/day	§	0.43 ± 0.13	§	3.41 ± 0.38	¶	0.87 ± 0.09

*Results expressed in mmols/24 hrs.
†Reported originally as cysteic acid in refs 21, 23, and 24.
‡β-Aminoisobutyric acid.
§Undetectable.
¶Unquantifiable traces.
**Not measured.

RESULTS (Tables 1 and 2)

In our initial study of patients with severe psoriasis unresponsive to other treatment, 6-AzUrd-TA administered at high doses (400 mg/kg/day) caused unusual hyperaminoaciduria including β-alanine (21) and L-homocystine (22).

Subsequent clinical studies revealed that the urinary excretion of β-alanine and L-homocystine also occurs after administration of lower doses (50 and 250 mg/kg/day) of 6-AzUrd-TA, is not specific for psoriasis, and quantitatively is dependent on the dose of the drug administered (23,24). Of particular interest is the fact that this hyperaminoaciduria was detected in two patients with mycosis fungoides at the time of remission achieved during 6-AzUrd-TA treatment (24).

Changes in amino acid metabolism caused by 6-AzUrd-TA were then studied in experimental animals; the rabbit was found to be a suitable model (24). In six animals studied, the hyperaminoaciduria was reproduced. It started at 5 (median) hours (range, 4–9) and reached its maximum at 15 (median) hours (range, 6–22) after oral administration of a single dose of 500 mg/kg of 6-AzUrd-TA (24). The amino acids β-alanine, β-aminoisobutyric acid, and L-homocystine were also detected in the serum of treated animals after repeated doses of 6-AzUrd-TA (25).

The biochemical effects of 6-AzUrd-TA were studied

in seven patients with progressive systemic sclerosis treated with therapeutic intent, and were found in all of them while they were receiving 9 g of 6-AzUrd-TA daily. In addition to the appearance of L-homocystine and β-alanine, significantly increased levels of L-methionine, L-threonine, and L-histidine were found (26).

These changes resemble those found in inborn homocystinuria, hyper-β-alaninemia, and hyperhistidinemia. Inborn hyperthreoninemia, to the best of our knowledge, has not yet been detected. Since the catabolism of all these amino acids is controlled by enzymes which utilize pyridoxal phosphate as coenzyme, inhibition of pyridoxal phosphate by 6-AzUrd-TA or by one of its metabolites was suggested as an explanation of these changes (26). This indirect evidence is supported by results on in vivo inhibition of two enzymes using pyridoxal phosphate as a coenzyme: rat liver cystathionine synthase (Slavik M, unpublished results) and serum histaminase in man (Baylin S, unpublished results) after repeated administration of 6-AzUrd-TA. The methods previously described (27,28) were used for the determination of the enzyme activity.

DISCUSSION

This paper summarizes results from our studies which indicate that 6-AzUrd-TA administration causes changes in amino acid metabolism, both in experimental animals and in man. We have previously reported that these changes are not derived from the 6-AzUrd-TA molecule itself as catabolites (21).

Structurally a pyrimidine nucleoside analog and functionally an inhibitor of the pyrimidine de novo synthesis, 6-AzUrd-TA represents an anticancer drug which causes profound changes in amino acid metabolism and is neither an amino acid-depleting enzyme nor, by origin, an amino acid antimetabolite. There are questions, however, that remain to be answered. What significance do these changes have and are they relevant to cancer treatment? Let us discuss them from the aspects of both clinical activity and toxicity.

Since most of our studies report 6-AzUrd-TA-induced changes in amino acid metabolism in patients with nonneoplastic diseases, there are not adequate data for their correlation with the antitumor effect. However, limited information is available on two patients with mycosis fungoides who were treated with daily doses of 250 mg/kg of 6-AzUrd-TA. Both of these patients were in partial remission when typical hyperaminoaciduria was detected (24). 6-AzUrd-TA effectiveness has also been reported in the treatment of other neoplastic diseases, including polycythemia vera (13), erythroleukemia (12), and trophoblastic malignancies (10,11). Although amino acid changes have not been studied in these diseases during 6-AzUrd-TA treatment, it is very likely that they occur. Their correlation with the antitumor effect would provide an interesting approach for studying the significance of these amino acid alterations to the biology of the disease.

6-AzUrd-TA clinical toxic effects involve bone marrow (4) and occasionally central nervous system (CNS) and thromboembolic episodes (7,14).

The bone marrow toxicity is moderate (4,5), predictable (4), and reversible (5), and results from the 6-AzUrd-TA interference with pyrimidine de novo synthesis (5).

The CNS toxicity is thought to be caused by the trace amounts of 6-azauracil, formed from 6-azauridine. However, the abnormalities of amino acid metabolism may offer an alternative explanation, since, in the inborn errors, both L-homocystine and β-alanine have been found toxic to the CNS (29,30).

Thromboembolic episodes have been the most serious side effect of 6-AzUrd-TA treatment and caused its withdrawal from the market; their origin remained unexplained. The patients with inborn L-homocystinuria are also known to have a high incidence of thromboembolic episodes, which are the major cause of death (29). The effect of L-homocystine, which normally does not appear in plasma, on the vascular endothelium with the subsequent formation of thrombi has been postulated to be responsible for the high incidence of vascular occlusive complications in this inborn disease (29,31).

In our study (26), only one of seven patients with progressive systemic sclerosis experienced a thrombotic episode during 6-AzUrd-TA treatment. High serum levels of L-homocystine were detected at the time of this episode, which subsided after the drug was discontinued (Slavik M, unpublished results).

These results suggest that drug-induced homocystinemia may be the possible explanation for the thrombotic episodes in 6-AzUrd-TA-treated patients. This hypothesis is currently being tested in a prospective study which compares the L-homocystine levels with the incidence of thrombotic episodes in patients with psoriasis treated with 6-AzUrd-TA (32). The preliminary results appear to confirm the hypothesis and suggest that monitoring of the L-homocystine levels may be a useful approach for the prevention of the thromboembolic side effects.

Our results also indicate that anticancer drugs other than amino acid analogs or amino acid-depleting enzymes may cause significant changes in amino acid metabolism, which may be related to the therapeutic or adverse effects of the drug. Their detection and elucidation may represent an alternate method for studying the relevance of amino acid changes to the biology and rational treatment of cancer.

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Biologic and Antineoplastic Effects of Enzyme-Mediated In Vivo Depletion of L-Glutamine, L-Tryptophan, and L-Histidine [1,2]

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SUMMARY

Novel enzymes, capable of depleting L-glutamine plus L-asparagine, L-tryptophan, or L-histidine, were purified from soil isolate organisms. L-Glutaminase-L-asparaginase from *Pseudomonas* 7A demonstrated substantial antineoplastic activity against a variety of L-asparaginase-resistant leukemias (L1210, EARAD/1/AR, and C1498), an ascites tumor (Taper liver tumor), and solid tumors (B16 melanoma and Walker 256 carcinosarcoma). *Pseudomonas* 7A L-glutaminase-L-asparaginase (PGA) is a considerably more potent antitumor agent than *Acinetobacter* L-glutaminase-L-asparaginase. An important characteristic of L-glutaminase-L-asparaginase therapy is that tumors do not develop resistance to this enzyme as they do to *Escherichia coli* L-asparaginase (EC-2). Resistance to EC-2 by EARAD/1 leukemia cells developed in two treatment generations. By contrast, after ten treatment generations with PGA, EARAD/1 leukemia cells were just as sensitive to both L-glutaminase-L-asparaginase and EC-2 as the parent tumor. Combination therapy for L1210 leukemia with L-glutaminase-L-asparaginase and methotrexate resulted in delayed development of tumor resistance to the antimetabolite from generation 5 to 14 and in lower host toxicity to methotrexate. A > 90% increase in median survival time was obtained against the solid form of Ehrlich carcinoma with a combination of PGA and azaserine, whereas either drug alone caused a < 15% increase in survival time. Indolyl-3-alkane α-hydroxylase, which attacks the side chain of L-tryptophan, serotonin, and a variety of other 3-substituted indole compounds, caused marked depletion of L-tryptophan and serotonin in body fluids and certain tissues. This enzyme exhibited significant antineoplastic activity against a variety of mouse tumors: Meth A sarcoma, Ehrlich carcinoma, and Taper liver tumor. Urinary excretion of serotonin and 5-hydroxyindoleacetic acid decreased to normal levels in P815 ×2 mastocytoma-bearing mice following treatment with indolyl-3-alkane α-hydroxylase. An L-histidase, which has near-optimal activity in the physiologic pH range and a K_m value of 1 mM, was isolated from a *Corynebacteriaceae* soil organism. This enzyme is inhibited 85% by 0.1 mM EDTA. This inhibition was reversed by 1 mM Mn^{++}, Zn^{++}, or Cu^{++}, but not by Mg^{++} or Ca^{++}. The plasma half-life of *Corynebacteriaceae* L-histidase in mice was about 8 hours. Treatment of tumor-bearing mice with 500 IU of L-histidase/kg/day maintained plasma L-histidine at unmeasurably low levels (< 3 nmols/ml) and resulted in inhibition of total packed cell volume of the ascitic forms of Ehrlich carcinoma and Meth A sarcoma.

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The antineoplastic effect of L-asparaginase against experimental and human lymphocytic leukemias has been well-documented (1,2). L-Glutamine, like L-asparagine, is a nonessential amino acid in the diet of man. L-Glutamine participates in a wide variety of metabolic reactions in mammalian cells (3). When compared with other tissues, certain tumor cells appear to operate at a marginal level of L-glutamine availability as a result of a slow rate of synthesis and a rapid utilization (4,5). L-Glutaminase-L-asparaginase has demonstrated antitumor activity against a much broader spectrum of neoplasms than L-asparaginase alone (6–10).

There are conflicting reports in the literature concerning the antineoplastic effects of maintaining tumor-bearing hosts on synthetic diets deficient in selected "essential" amino acids (11). We have found that even after 2–3 weeks on amino acid-deficient diets, only partial deficiencies of individual amino acids were observed in plasma of mice. Studies with three synthetic diets (lacking the specific amino acids L-phenylalanine and L-tyrosine, L-isoleucine, or L-threonine) in tumor-bearing mice revealed that tumors were able to grow at the expense of carcass weight and there was very little or no increase in survival time (12). It should be possible to effectively block availability to the tumor of an essential amino acid derived from catabolism of normal tissue protein by injection of an appropriate amino acid-degrading enzyme. An important advantage of treating tumor-bearing hosts by deprivation of an essential amino acid, as opposed to a nonessential amino acid, is that tumor resistance to deprivation of an essential amino acid is not expected to occur.

Of the essential amino acids, L-tryptophan is unique in the diversity of functions it can provide other than as a constituent of protein (13). Thus, deprivation of this amino acid may be particularly deleterious to cellular proliferation. Selective inhibition of tumors may result from enzyme-mediated depletion of L-tryptophan for short periods if the tumor cells have a greater requirement for this amino acid than normal tissues. Woolley et al (14) reported that limitation of L-tryptophan in the growth medium of L1210 leukemia cells causes a rapid fall in DNA and histone synthesis which precedes the decline in whole cellular protein synthesis.

Although L-histidine had been identified as a dietary essential amino acid for growing animals of several species, its necessity in the diet of adult humans has only recently been established (15). Eagle and co-workers (16) found that L-histidine was required in tissue culture by human carcinoma of the cervix (HeLa strain), epidermoid carcinoma of the nasopharynx (KB strain), and monocytic leukemia (Osgood's J-111). Ohnuma et al (17) found that omission of L-histidine or L-glutamine from the culture medium resulted in a marked decrease in uridine uptake by human acute myelocytic and lymphocytic leukemia cells.

In this paper we describe a variety of biologic and antineoplastic effects of novel L-glutamine- and L-tryptophan-degrading enzymes which were isolated in our laboratory specifically for therapeutic evaluation (18–22). We also describe the partial purification and characteristics of a novel L-histidase obtained from a *Corynebacteriaceae* soil isolate organism.

MATERIALS AND METHODS

Enzymes

The L-glutaminase-L-asparaginase enzymes were isolated from *Acinetobacter glutaminasificans* (ATCC-27197) and *Pseudomonas* 7A (ATCC-29598). These enzymes were purified to homogeneity as previously described (18,19). The specific activity of the pure *Acinetobacter* (AGA) enzyme was 180 ± 20 IU of L-glutaminase and 150 ± 16 IU of L-asparaginase/mg of protein. The specific activity of the pure *Pseudomonas* 7A (PGA) enzyme was 160 ± 15 IU of L-glutaminase and 80 ± 8 IU of L-asparaginase/mg of protein. When only one activity is given for an L-glutaminase-L-asparaginase enzyme, it refers to its L-glutaminase content.

Purified *Escherichia coli* L-asparaginase (EC-2) was obtained from Merck Sharp & Dohme Laboratories, West Point, Pa. The specific activity of the enzyme was approximately 300 IU/mg of protein.

Indolyl-3-alkane α-hydroxylase (IAH), which attacks L-tryptophan and other indole-3-alkanes, was purified to homogeneity from *Pseudomonas* XA (ATCC-29574) as previously described (21). The specific activity of pure enzyme was approximately 14 IU/mg of protein.

L-Histidase (L-histidine ammonia-lyase) was isolated from a laboratory gram-variable soil isolate organism (H-1) which has been tentatively classified as a *Corynebacteriaceae*. This L-histidase is inducible as indicated by a 20-fold increase in specific activity when the bacteria were grown in a minimal medium containing 1.5% L-histidine·HCl·H_2O, compared with growth in trypticase soy broth. Urocanic acid, a deaminated product of L-histidine, also served as an inducer. Yeast extract, at 1%, repressed L-histidase induction by > 60%. Bacteria were routinely grown at 30°C with aeration in Erlenmeyer flasks on a rotary shaker or in a fermentor. The cells were harvested by centrifugation at the end of the exponential phase of growth and stored at –75°C until needed. The cell recovery was 6–7 g of packed cells/liter. Each liter of growth medium contained 15 g of L-histidine·HCl·H_2O, 2 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 6 mg each of $FeCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnCl_2$, $CuSO_4 \cdot 5H_2O$, $CaCl_2 \cdot 2H_2O$, and NaCl. The pH was adjusted to 7.0.

Bacterial cells were suspended in 2 volumes of 0.05 M potassium phosphate buffer (pH 7.2) and were sonically disrupted with a Bronwill Biosonik IV sonifier with three 3-minute bursts. The sonicate was clarified by centrifu-

gation. Partial purification (about tenfold) of L-histidase was attained by ammonium sulfate precipitation (45%–60% saturation) followed by chromatography on a DEAE-Sephadex column which had been equilibrated in 0.04 M potassium-phosphate buffer, pH 8. Protein was eluted with a 0–1 M linear KCl gradient. The specific activity of the partially purified L-histidase was 2.1 IU/mg of protein.

Protein Determination and Enzyme Assays

Protein was determined by the method of Lowry et al (23) using crystalline bovine serum albumin as the standard. L-Glutaminase (19), L-asparaginase (19), and IAH (21) activities were determined as previously described. For determination of L-histidase activity, 0.1 ml of diluted enzyme solution was added to 2.9 ml of 5 mM L-histidine solution in 0.1 M potassium-phosphate buffer, pH 7.2. The reaction was followed in a quartz cuvette at 30°C by taking readings at 277 nm in a spectrophotometer every minute for 5 minutes. The increase in absorbance is caused by the formation of urocanic acid from L-histidine. One IU of L-histidase is the amount of enzyme needed to produce 1 μmol of urocanic acid/minute under the conditions of the assay.

Amino Acid Determinations

L-Glutamine, L-asparagine, L-glutamate, L-aspartate, and L-histidine concentrations were determined on a Beckman model 119 amino acid analyzer using lithium citrate buffers for the acidic and neutral amino acids (24,25). Blood for determination of plasma amino acids was obtained from mice by the orbital-bleeding technique (25). Special care was taken to minimize amino acid alterations during the processing of samples for amino acid analysis (25). L-Tryptophan was assayed fluorometrically by the method of Denckla and Dewey (26), slightly modified by Bloxam and Warren (27).

Serotonin and 5-Hydroxyindoleacetic Acid Determinations

Serotonin in urine was assayed by using Amberlite IRC-50 columns, as described by Lovenberg and Engelman (28). 5-Hydroxyindoleacetic acid in urine was first isolated by differential solvent extraction, and was then estimated by the 1-nitrosonaphthol color assay. The procedure, as described by Lovenberg and Engelman (28), was scaled down 30-fold to permit assay of 0.2 ml of urine. Slight modifications used were the omission of the dinitrophenylhydrazine step for removing excess α-keto acids and the use of unwashed ether so that antioxidant activity would be retained in the ether. The recovery rate of internal standard was usually 50%.

Animals and Tumors

BDF_1 (C57BL/6 × DBA/2) mice (obtained from the A. R. Schmidt Co, Madison, Wisc) were the hosts for the following tumors: Taper liver tumor, Ehrlich ascites tumor, B16 melanoma, L1210/V leukemia (L1210), and myeloid C1498 leukemia (C1498). EARAD/1 and its subline EARAD/1/AR, which is resistant to L-asparaginase, were grown in female $B6AF_1/J$ (C57BL/6J × A/J) mice. Meth A sarcoma was grown in (BALB/cJ × C57BL/6J)F_1/J mice, and P815 ×2 mastocytoma was grown in DBA/2J mice. All three hybrids were purchased from the Jackson Laboratory, Bar Harbor, Me. Walker 256 carcinosarcoma was grown in CDF_1 rats that were purchased from the Charles River Breeding Laboratories, Wilmington, Mass. The tumors used in this study were either carried by us or obtained from other investigators at our Institute. Only C1498 was purchased from the Jackson Laboratory.

For the growth of the ascites tumors, 10^7 cells were injected ip, except for L1210 (10^5 cells) and EARAD/1 (4×10^5 cells). For the development of solid tumors, 10^7 cells were injected sc into the right axillary region. B16 melanoma and Walker 256 carcinosarcoma tumors were developed by transplantation of small fragments into the right axillary region. The viability of the ascites cells was determined by the dye-exclusion method. Cells were counted in 0.025% trypan blue-0.85% NaCl solution. The ascitic fluids used for tumor implantation contained at least 95% viable cells.

It is known that certain enzymes, eg, EC-2 or AGA (25), are cleared at a considerably slower rate from the peripheral blood of mice that are infected with the lactate dehydrogenase-elevating virus (LDH virus) than in corresponding virus-free mice. Most transplantable mouse tumors have been found to be infected with the LDH virus (29). Therefore, to ensure uniform experimental conditions, we injected ip 0.1 ml of 1:10 diluted plasma from LDH virus-infected mice into all mice, usually 3 days before the start of an experiment.

Chemotherapeutic Tests

The day of tumor transplantation was Day 0. The various treatment schedules are indicated later in the text or in the tables. The effect of treatment on the ascites tumors and leukemias was evaluated on the basis of the average total packed cell volume (TPCV) and/or the median survival time (MST). The difference in the weight of the mouse before tumor inoculation and its weight after removal of ascitic fluid represented the animal weight change (AWC). After the ascitic fluid was centrifuged at 10,000 rpm for 7 minutes in an International hematocrit centrifuge, the TPCV was calculated as the volume of ascitic fluid multiplied by the fraction of the volume occupied by the packed tumor cells.

The sc implanted tumors were measured in two perpendicular diameters. The average tumor diameters (ATD) were pooled for the various groups of animals. The most significant parameter in evaluating antitumor efficacy is the MST; however, measurements of TPCV and ATD were used as secondary parameters. All tumors used, with the exception of EARAD/1, were insensitive to EC-2. Daily ip injections of EC-2 at a dose of 250 IU/kg for 7 days caused a < 20% increase in MST for L1210 or C1498 leukemia. The same dose produced a < 20% decrease in the ATDs of the solid forms or the TPCVs of the ascitic forms of Taper liver tumor, Ehrlich ascites carcinoma, and Meth A sarcoma (Schmid F, and Roberts J, unpublished results). B16 melanoma was found to be resistant to L-asparaginase by Sugiura (30).

RESULTS

L-Histidase

The bacterial L-histidase described in this report exhibits a relatively broad pH-activity profile curve between pH 7.2 and 9 (fig 1). The K_m value of this enzyme was determined to be approximately 1 mM. Enzymatic activity was inhibited 85% by EDTA (0.1 mM). This inhibition was reversed by 1 mM Mn^{++}, Zn^{++}, or Cu^{++}, but not by Mg^{++} or Ca^{++}.

The circulating half-life of the *Corynebacteriaceae* L-histidase in BDF_1 mice that had been infected with LDH virus was about 8 hours. Single ip injections of 400 IU of L-histidase/kg lowered plasma L-histidine from pretreatment levels of about 100 nmols/ml to unmeasurably low levels (< 3 nmols/ml) within 1 hour and maintained these low levels for 12–24 hours. Preliminary studies indicated that a dose of 250 IU of L-histidase/kg injected ip twice daily for 3 days produced marked reduction of TPCV of Ehrlich ascites carcinoma (93% reduction) and Meth A ascites sarcoma (77% reduction).

L-Glutaminase-L-Asparaginase

Previous studies have revealed that AGA, which has a relatively short biologic half-life, was effective against a variety of L-asparaginase-resistant ascites tumors in mice but had little or no effect against solid tumors (6,7). Various chemical modifications were tried to prolong the plasma half-life of AGA and to increase its effectiveness against solid tumors (20). Succinylation or glycosylation of the enzyme increased the plasma half-life sufficiently for large animal antitumor testing in leukemias and ascites tumors, but did not increase the effect of the enzyme against solid tumors (20,31). Recently, we isolated a new L-glutaminase-L-asparaginase from a soil organism, *Pseudomonas* 7A (19). This enzyme has an unusually long biologic half-life (19). PGA demonstrated substantial antineoplastic effectiveness against a variety of leukemias

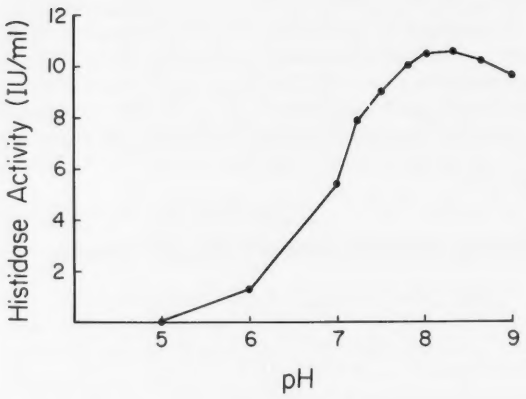

FIGURE 1.—Effect of pH on activity of *Corynebacteriaceae* L-histidase.

(L1210, EARAD/1, and C1498), ascites tumors (Taper liver), and solid tumors (Walker 256 carcinosarcoma and B16 melanoma) (table 1). PGA is a considerably more potent antitumor agent than AGA. PGA lowered free L-glutamine and L-asparagine in solid tumor tissue to a much lower level than AGA (table 2).

One of the most striking findings relevant to L-glutaminase therapy is that tumors do not seem to develop resistance to this enzyme as they do with L-asparaginase or other antitumor drugs. PGA administered for ten to 14 treatment generations to mice bearing L1210 or EARAD/1 leukemia had little effect in selecting or inducing a resistant line. Resistance by EARAD/1 leukemia cells to EC-2 developed in two treatment generations (table 3). By contrast, after ten treatment generations with PGA, EARAD/1 leukemia cells were just as sensitive to both L-glutaminase-L-asparaginase and EC-2 as the parent tumor (table 4). It is also noteworthy that combination therapy for L1210 leukemia with PGA and methotrexate substantially delayed development of tumor resistance to methotrexate (table 5). Combination therapy with methotrexate and L-glutaminase-L-asparaginase resulted in lower host toxicity due to methotrexate (table 6).

Studies are currently underway in our laboratory to determine whether co-administration of L-glutamine analogs with L-glutaminase improves their therapeutic effectiveness against solid tumors. It might be expected that selected L-glutamine analogs may have greater antineoplastic activity in tumor-bearing hosts when the available pools of L-glutamine have been drastically depleted by co-administration of L-glutaminase. Initial studies indicated that treatment with PGA or azaserine alone produced a < 15% increase in MST in mice bearing the solid form of Ehrlich carcinoma. Combination treatment with L-glutaminase and azaserine, however, prolonged the MST of tumor-bearing mice by > 90% (table 7).

TABLE 1.—Antitumor effect of L-glutaminase-L-asparaginase*

Tumor	Enzyme	Dose (IU/kg)	Treatment days	Day 14 ATD (cm) Treated	Day 14 ATD (cm) Control	Day 14 AWC (g) Treated	Day 14 AWC (g) Control	MST (days) Treated	MST (days) Control
B16 melanoma	PGA	90	1–7	1.09	1.63	−0.5	+1.1	36	25
B16 melanoma	AGA	250	1–14	1.22	1.55	+0.2	+3.6	31	25
Walker 256 carcinosarcoma	PGA	200	1–7	0.5	2.03	+8.4	+38	—	—
Walker 256 carcinosarcoma	AGA	1000	1–7	1.60	2.05	+30	+38	—	—
C1498 leukemia	PGA	100	1–14	—	—	−0.7	+0.9	19	15
L1210 leukemia	PGA	125	1–6	—	—	−4.2	+2.8	14	9
EARAD/1 leukemia	PGA	75	1–10	0	1.56	−0.8	+0.6	> 60 (2 of 5)	15
EARAD/1 leukemia	AGA	250	1–10	0	1.56	−0.1	+0.6	29	15
EARAD/1/AR leukemia	PGA	75	1–10	0.32	1.75	−1.3	+3.1	24	16
Taper liver tumor	PGA	100	1–21	—	—	—	—	59 (2 of 5)	16
Taper liver tumor	AGA	125	1–21	—	—	—	—	42	16

*All tumors were implanted sc except Taper liver tumor which was implanted ip. Tumors were implanted on Day 0. There were 5 animals/group. *See* text for other experimental details.

TABLE 2.—Effect of L-glutaminase-L-asparaginase treatments on amino acid levels in EARAD/1 tumor*

Treatment	Free amino acid concentration (nmols/g of tissue) L-Glutamine	L-Asparagine
Control (saline)	198	203
AGA	157	94
PGA	61	39
EC-2*	950	26

*Tumor cells (4×10^6) were injected sc on Day 0. Ip injections of enzyme (200 IU/kg/day) were given for 3 days starting on Day 11, when the ATDs were approximately 1.1 cm. The tumors were excised on Day 15 for amino acid analyses. The values are averages of tumors from 3 mice.

TABLE 3.—Development of tumor resistance to L-glutaminase-L-asparaginase and to L-asparaginase

Enzyme*	Treatment generations until resistant EARAD/1	EARAD/1/AR	L1210
EC-2 (10 IU/kg/day × 7)	2	Resistant	Resistant
PGA (75 IU/kg/day × 7)	> 13	> 14	> 10

*The enzymes were injected ip in 7 daily injections starting 1 day after ip inoculation of tumor cells from the treated group of the previous generation. After every 2 or 3 treated generations, the sublines were tested for sensitivity to each enzyme.

TABLE 4.—Effect of L-glutaminase-L-asparaginase on EARAD/1 tumors*

	Day 14			
	ATD (cm)	AWC (g)	MST (days)	45-day survivors/ No. treated
EARAD/1				
Control	1.67	+1.1	15	0/5
PGA†	0	−0.6	43	0/5
EC-2‡	0.02	−0.2	> 52	3/5
EARAD/1/AR				
Control	1.69	+2.6	18	0/5
PGA†	0	−3.3	28	0/5
EC-2‡	1.80	+1.4	18	0/5
EARAD/1/7A (10)§				
Control	1.13	+3.4	18	0/5
PGA†	0	−1.2	42	0/5
EC-2‡	0	+1.8	> 47	5/5
EARAD/1/AR/7A (10)§				
Control	1.30	+2.7	18	0/5
PGA†	0	−0.7	29	0/5
EC-2‡	1.21	+1.9	18	0/5

*Tumor cells (4×10^5) were injected sc into $B6AF_1$ mice on Day 0; they were treated with enzyme on Days 1–6.

†PGA dose, 50 IU/kg/day of L-asparaginase and 100 IU/kg of L-glutaminase.

‡EC-2 dose, 50 IU/kg/day.

§Tumor sublines after 10 treatment generations with PGA.

TABLE 5.—Effect of L-glutaminase-L-asparaginase on development of resistance by L1210 tumor to antimetabolites

	Treatment generations until resistant	
Drug*	Drug alone	Drug + enzyme
Methotrexate	5	14
6-Thioguanine	2	2
Cytosine arabinoside	7	6

*6 daily ip injections of drugs were given starting 1 day after ip inoculation of 10^5 L1210 cells. The antimetabolites were injected at a ¼-LD10 dose 3 hrs after injection of PGA (100 IU/kg). The ¼-LD10 doses used were as follows: methotrexate, 0.85 mg/kg; 6-thioguanine, 0.73 mg/kg; and cytosine arabinoside, 15 mg/kg.

TABLE 6.—Effect of L-glutaminase-L-asparaginase on methotrexate toxicity*

Treatment	MST in days (range)
Saline (control)	10 (9–11)
PGA (100 IU/kg)	12 (11–13)
Methotrexate (18 mg/kg)	11 (9–12)†
PGA + methotrexate‡	27 (18–27)

*BDF_1 mice were injected with L1210 leukemia cells on Day 0 and treated every other day (Days 1–14).

†Toxicity deaths.

‡Methotrexate was injected 6 hrs after PGA.

Indolyl-3-alkane α-Hydroxylase

IAH is a newly described bacterial hemoprotein which catalyzes hydroxylation on the side-chain carbon adjacent to the indole ring of a variety of 3-substituted indole compounds, including L-tryptophan and serotonin (21). The plasma half-life of this enzyme is approximately 7 hours in normal mice and approximately 25 hours in tumor-bearing mice (21). Single injections of 100 units of IAH/kg lowered plasma L-tryptophan in Meth A sarcoma tumor-bearing mice from a pretreatment level of 76 to < 5 nmols/ml within 4 hours and maintained this low level for 24 hours. L-Tryptophan concentrations were also markedly lowered in plasma, brain, and lungs of normal mice following a single injection of 600 units of IAH/kg. There was only a small and transient drop in L-tryptophan levels in the liver and kidneys of enzyme-treated mice (figs 2 and 3).

Injections of 100 units of IAH/kg/day for 3 days resulted in a > 94% reduction of TPCV of the following ascites tumors: Meth A sarcoma, Taper liver tumor, and Ehrlich carcinoma (table 8). Daily injections of 100 units of IAH/kg for 8 days were well-tolerated by mice and treatment of Meth A sarcoma tumor-bearing mice with this enzyme greatly prolonged their MST (table 9). It had been reported that the extent of growth of P815 × 2 mastocytoma in DBA mice may be correlated with increases in urinary concentrations of 5-hydroxyindoles (32). In the present study we have observed marked increases in urinary ex-

TABLE 7.—Combination treatment of Ehrlich carcinoma with L-glutaminase-L-asparaginase and azaserine*

L-Glutaminase-L-asparaginase		Azaserine dose (mg/kg)	MST (days)	
Dose (IU/kg)	Treatment days		Treated	Control
60	1–9	—	48	42
—	—	2	48	42
60	1–9	2	81	42

*Ehrlich carcinoma cells (10^7) were injected sc into BDF_1 mice on Day 0. PGA was injected ip in the morning and azaserine was injected in the afternoon. There were 5 mice/group.

TABLE 8.—Effect of IAH on ascites tumors*

Tumor	TPCV (ml)		Treated/ control
	Treated	Control	
Ehrlich ascites carcinoma (BDF_1 mice)	0.05	0.91	0.06
Taper liver tumor (BDF_1 mice)	0.04	0.97	0.04
Meth A sarcoma (BALB/cJ × C57BL/6J)F_1/J mice	0.03	1.2	0.03

*Mice were injected ip with 10^6 (Ehrlich ascites carcinoma and Meth A sarcoma) or 10^7 (Taper liver tumor) cells on Day 0. Treatment with 100 units of enzyme/kg/day × 3 was started on Day 4. Average TPCV of tumors was determined on Day 7. There were 3 mice/group.

TABLE 9.—Effect of IAH on Meth A sarcoma*

Treatment (dose)	Days of injection	MST in days (range)	
		Treated	Control
IAH (50 units/kg)	3–10	26 (21–180+)	18 (17–24)
IAH (100 units/kg)	3–10	24 (19–38)	18 (17–24)
Cyclophosphamide (100 mg/kg)	2,16	14 (14–15)	15 (14–15)
IAH (200 units/kg)	1,6,9,11,14,16	29 (6†–44)	15 (14–15)
+ cyclophosphamide (100 mg/kg)	2,16		

*Meth A sarcoma cells (10^5) were injected ip into (BALB/cJ × C57BL/6J)F_1/J mice on Day 0. There were 5 mice/group. Cyclophosphamide was given to delay development of antibodies to the enzyme.

†1 mouse died from drug toxicity.

TABLE 10.—Effect of IAH on serotonin and 5-hydroxyindoleacetic acid excretion in P815 ×2 mastocytoma-bearing mice*

		Urinary excretion (μg/mouse/24 hrs)	
	Treatment	Serotonin	5-Hydroxy-indoleacetic acid
Normal mice	—	0.85	16.2
Tumor-bearing mice	Saline	60	95.7
Tumor-bearing mice	IAH	0.85	16.7

*P815 ×2 mastocytoma cells (10^6) were injected ip into male DBA/2 mice on Day 0. IAH (150 units/kg) was injected ip on Days 2, 3, 4, and 6. Urine was collected in metabolism cages on Days 6 and 7. There were 4 mice/group.

cretion of serotonin and 5-hydroxyindoleacetic acid in P815 ×2 tumor-bearing mice. These indole derivatives dropped to normal concentrations when tumor-bearing mice were treated with IAH (table 10).

DISCUSSION

Results obtained in this study indicate that the newly described L-glutaminase-L-asparaginase derived from the soil isolate organism *Pseudomonas* 7A is a considerably more potent antineoplastic agent than AGA. L-Glutaminase-L-asparaginase exhibited substantial antitumor effectiveness against a variety of neoplasms which are resistant to EC-2, including myelogenous leukemia, carcinoma, sarcoma, and melanoma. L-Glutaminase-L-asparaginase is also effective against L-asparaginase-sensitive

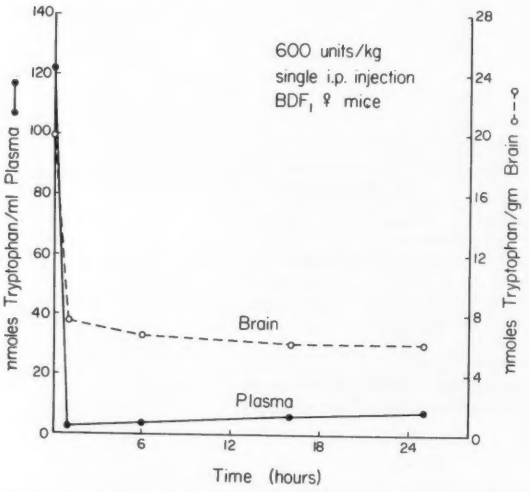

FIGURE 2.—Effect of IAH on mouse plasma (●——●) and brain (○----○) L-tryptophan concentrations. Female BDF_1 mice were injected ip with 600 units of IAH/kg. Each point represents the average value for 2 animals.

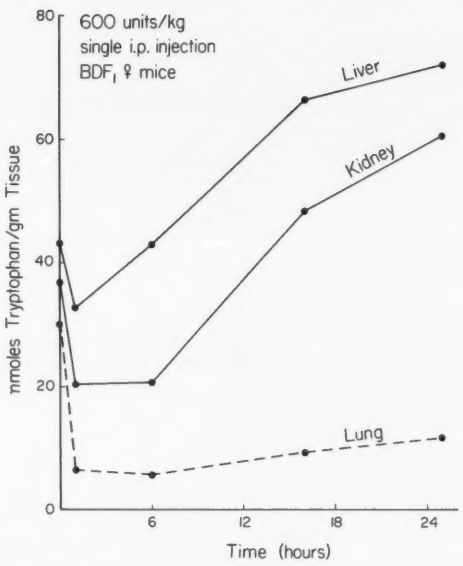

FIGURE 3.—Effect of IAH on mouse liver, kidney, and lung L-tryptophan concentrations. The experimental conditions were the same as for fig 2.

lymphocytic leukemias. However, unlike L-asparaginase, L-glutaminase-L-asparaginase treatment did not result in development of tumor resistance to the drug. It may, therefore, be advantageous to treat even L-asparaginase-sensitive tumors with L-glutaminase-L-asparaginase. It was also noted that co-administration of L-glutaminase-L-asparaginase substantially delayed development of tumor resistance to methotrexate, and also lowered host toxicity to the antimetabolite. The rationale for using an L-glutamine analog in combination with L-glutaminase is to compensate for the residual L-glutamine that may be produced by normal cells surrounding the tumor, which cannot be depleted by the circulating enzyme. Experiments showed that combination treatment with L-glutaminase-L-asparaginase and azaserine prolonged the MST of mice bearing the solid form of Ehrlich carcinoma by > 90%. Either drug used alone caused a < 15% increase in survival time.

Because L-glutamine, a nonessential amino acid, is synthesized by normal cells and enters the bloodstream, simple dietary deprivation would not produce L-glutamine deficiency in the host. For therapeutic effectiveness, L-glutamine must be continuously degraded in the body fluids by parenteral administration of L-glutaminase. Diets which are deficient in essential amino acids were first used for tumor control in animals nearly 60 years ago (33), and work in this area followed in a number of laboratories (11) yielding conflicting results. Relatively

few studies of this nature have been conducted in man with malignancies because the synthetic diets were found to be cumbersome, complex, and unpalatable. It is expected that administration of specific amino acid-degrading enzymes will circumvent these problems and also block availability to the tumor of an essential amino acid derived from catabolism of normal tissue protein. Prolonged deprivation of an essential amino acid will be toxic to the tumor-bearing host. However, deprivation of essential amino acids for short periods of time (2–3 weeks) appears to be well-tolerated by the host.

We describe here the isolation of a novel L-histidase from a *Corynebacteriaceae* soil organism. This enzyme has a relatively broad pH-activity curve between pH 7.2 and 9, with nearly 75% of maximum activity at pH 7.2. Although this enzyme is being developed primarily as a potential new antineoplastic agent, it may also be ideally suited for treatment of the inborn error of L-histidine metabolism known as L-histidinemia. Abuchowski et al (34) have reported that attachment of polyethylene glycol to certain enzymes rendered the proteins nonimmunogenic. If bacterial L-histidase can be rendered nonimmunogenic, it should be possible to administer the enzyme over extended treatment periods.

The newly described enzyme, IAH (21,22), has significant antineoplastic activity against a variety of experimental tumors. Of particular interest was the finding that urinary excretion of serotonin and 5-hydroxyindoleacetic acid decreased to normal levels following treatment of mastocytoma-bearing animals with the enzyme (table 10). IAH, which can lower L-tryptophan and serotonin levels in body fluids and some tissues, may be useful for treating human carcinoid tumors as well as a variety of other disorders which are characterized by elevated serotonin levels. IAH's ability to induce marked hypothermia in mice (up to 11°C drop in rectal temperature) when administered at a high dose (> 300 units/kg) may be an especially useful characteristic. Concomitant with the enzyme-induced hypothermia, serotonin and L-tryptophan concentrations were markedly lowered in the brains of treated mice. By contrast, only minor fluctuations were noted in brain norepinephrine and dopamine concentrations in animals treated with high doses of IAH (35).

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Influence of L-Tyrosine Phenol-lyase on the Growth and Metabolism of B16 Melanoma [1]

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SUMMARY

Administration of L-tyrosine phenol-lyase inhibited growth of established B16 melanomas in BDF_1 mice. Under the conditions used, the enzyme exerted a cytostatic effect on the tumors. The addition of L-tyrosine phenol-lyase to growing melanoma cultures blocked cell division and caused an accumulation of cells in the G_1 (G_0) phase of the cell cycle. While the uptake of L-leucine and thymidine in treated cultures was suppressed, the most pronounced effect of L-tyrosine phenol-lyase was a rapid and near-complete cessation of uridine uptake and incorporation. This block in RNA synthesis was also observed in human fibroblasts treated with L-tyrosine phenol-lyase.

[Cancer Treat Rep 63:1055–1062, 1979]

L-Tyrosine and L-phenylalanine are of special importance to pigmented malignant melanocytes because of their dual requirement for these amino acids as precursors in both protein and melanin synthesis. It has been known for some time that dietary depletion of these amino acids has resulted in therapeutic benefits in both animals (1–3) and man (4) with melanoma. The difficulties and limitations inherent in prolonged dietary therapy (4) have prompted us to examine the potential applicability of L-tyrosine- and L-phenylalanine-degrading enzymes in melanoma. L-Tyrosine phenol-lyase (TPL), an enzyme elaborated by *Erwinia herbicola* and certain other *Enterobacteriaceae* (5), was found to inhibit the growth of B16 melanoma implanted in mice (6). We report here on the in vitro and in vivo activities of this enzyme and on metabolic changes in enzyme-treated melanocytes.

METHODS AND MATERIALS

In Vivo Procedures

TPL was purified from *Erwinia herbicola* (ATCC-21434) and was assayed as previously described (6). The purified enzyme had a specific activity of 1.4 IU/mg of protein, and was homogeneous on gel electrophoresis. The lyophilized enzyme was routinely reconstituted just prior to use with 0.01 M potassium-phosphate buffer, pH 7.4, containing 0.2 mg/ml of pyridoxal phosphate (PLP).

C57BL/6 × DBA/2F_1 (BDF_1) female mice, weighing 17–21 g, were obtained from the Charles River Breeding Laboratory, Wilmington, Mass. In order to avoid variability due to the presence or absence of LDH virus infection in the mouse population (7,8), all mice were intentionally infected with this benign virus 2–3 days prior to experimentation (6). The LDH virus by itself does not significantly affect the growth rate of the pigmented B16 melanoma (9).

In Vitro Procedures

B16 melanoma tumors were routinely maintained as solid tumors in the subcutaneous hip tissue of BDF_1 mice. Tumors were explanted into Falcon tissue culture flasks (25 cm^2); only the first subcultures were used for experimental purposes. Cells were given 48 hours to recover, adhere, and stabilize before treatment was initiated. Cultures were supplied with fresh medium and were treated on the second and fourth day after subculture. A second cell line used was normal human fetal skin fibroblasts (ATCC CRL-1106). These cells were in their sixth passage at the time the experiments were performed.

Cell viability was determined by the trypan blue dye exclusion test, and data are presented as the mean value of four replicate cultures ± 1 SD. Cells were grown in

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medium 199 (BBL) or Eagle's minimum essential medium (GIBCO). All culture media, unless noted otherwise, were supplemented with 20% whole fetal calf serum, 1 mM L-glutamine, 1 mM pyruvate, 1% (vol/vol) nonessential amino acid mixture (GIBCO), and 1% (vol/vol) penicillin streptomycin mixture (GIBCO). Pyruvate, a product of the TPL-catalyzed reaction, was not added to the medium used subsequent to enzyme addition. Treated cultures received 0.1 ml of the enzyme in 0.01 M potassium-phosphate buffer, pH 7.4, containing 0.2 mg/ml of PLP, whereas control cultures received 0.1 ml of the buffer-PLP solution. L-Tyrosine levels were determined by automated amino acid analysis through the courtesy of the Harborview Medical Center Clinical Laboratories, Seattle, Wash.

For the precursor-uptake studies, cultures were grown in Costar trays. Cells were inoculated at 1×10^5 cells/ml and were incubated for 2 days before studies were initiated. [^{3}H]-Thymidine (2.0 curies/mmol), [^{3}H]-uridine (47.9 curies/mmol), and L-[^{3}H]-leucine (5 curies/mmol) (obtained from New England Nuclear Corp, Boston, Mass) were added separately to each of four wells to give a final concentration of 2 microcuries/ml. After 2 hours, the media were removed by aspiration and the uptake of precursor was determined essentially as described by Pardee (10). For cell cycle analysis, replicate B16 melanoma cultures were treated for 48 hours with TPL. Cells were then gently washed with 0.05% trypsin in 0.029 mM EDTA; this medium was decanted, and then the cells were incubated with the same medium for an additional 5–10 minutes at 37°C. Neutralization solution (10 ml) (11) was added, and the suspension was aspirated into and expelled from a pipet several times, then chilled on ice for 5 minutes, and centrifuged at $150 \times g$ for 5 minutes at 4°C; supernatant then was decanted. Cells were resuspended directly into hypotonic propidium iodide solution as described by Crissman et al (11,12). Samples were analyzed by flow microfluorometry at a laser excitation wave-length setting of 488 nm through the courtesy of the Department of Pathology, University of Washington.

RESULTS

L-Tyrosine Phenol-lyase Effects of In Vivo Growth

As shown in table 1, TPL administration resulted in significant suppression of B16 melanoma growth. No overt toxic manifestations were noted in treated animals and weight changes essentially paralleled tumor growth. Cessation of enzyme treatment, however, resulted in renewed growth of the tumor and, with the relatively short treatment protocol used, significant regression of tumor growth was not observed in these studies.

TABLE 1.—TPL inhibition of B16 melanoma growth*

Trial No.	Day	T/C†
1	1	0.54
2	1	0.39
1	3	0.29
2	3	0.13‡
1	5	0.20‡
2	5	0.21‡
1	7	0.26‡
2	7	0.27‡
1	9	0.36‡
2	9	0.39‡
1	11	0.53
2	—	—

*Mice with tumors were treated for 6 days starting on Day 0, 7–10 days after tumor implantation, with 100 IU/kg of TPL every 12 hrs ip. Control mice were similarly injected with 0.2 ml of PLP (50 μg/ml) in 0.01 M phosphate buffer, pH 7. In trial 2, all mice were fed a vitamin B6-enriched diet (1 mg of pyridoxine and 1 mg of PLP/g of chow) and, in addition, received 1 mg of PLP ip at the time of treatment. There were 10 animals/experimental group except for the enzyme-treated group in trial 2 which contained 8 animals.

†Volume of tumors in treated animals/volume of tumors in control animals.

‡Significant at $P < 0.01$; all other values were $P > 0.01$.

L-Tyrosine Phenol-lyase Effects on In Vitro Growth

Low doses of TPL suppress in vitro growth of B16 melanoma (figs 1–3). Cells apparently completed approximately one cell division after treatment with the enzyme, but further division was dependent on the dose of enzyme used (fig 1). It is expected that the principal action of TPL is to deprive growing cultures of essential L-tyrosine. However, it is also possible that TPL toxicity is due to some direct effect on the cell membrane or activity on other nutrients, or that products of the enzyme catalysis such as phenol are responsible for the block in cell division. Figure 2 shows melanoma cell division in media pretreated with TPL followed by removal of the enzyme by ultrafiltration. Cells grown in pretreated media were never directly exposed to the enzyme. Also examined were media without L-tyrosine, L-tyrosine-free media treated with TPL and filtered as above, and media containing active enzyme. In each case, cells divided approximately once before inhibition of growth was observed. TPL apoenzyme only slightly inhibited cell division (fig 3). The small amount of activity of the apoenzyme preparation (< 2% of holoenzyme activity) is probably responsible for the observed effect. Phenol, at the maximum possible concentration achieved through enzyme catalysis (42 μg/ml), was nontoxic. The time course of L-tyrosine depletion and TPL stability in media is shown in figure

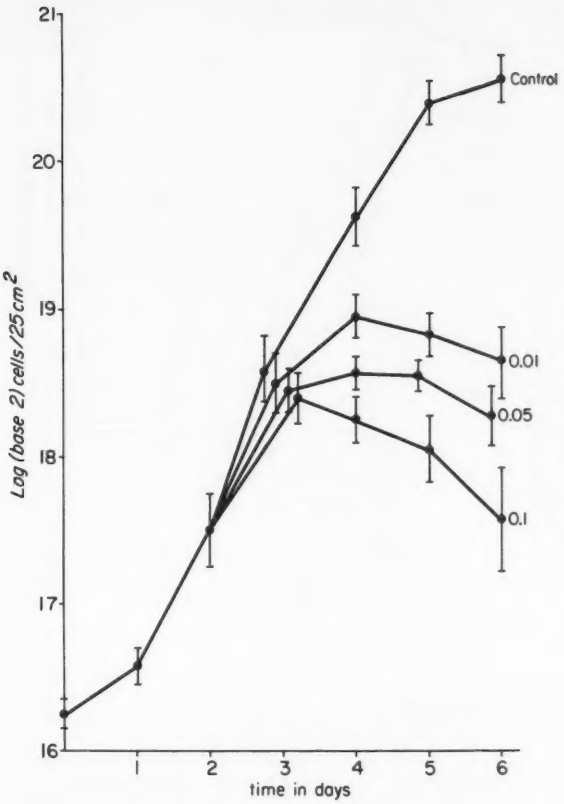

FIGURE 1.—Dose response in growth of cultured mouse melanoma cells treated with TPL. Cultures were grown in medium 199. Cells were treated on Days 2 and 4. Cell counts are expressed in log to the base 2 units so that an increase of 1 unit on the log scale corresponds to the equivalent of 1 division by each cell. Enzyme concentrations in treated cultures are expressed as IU/ml.

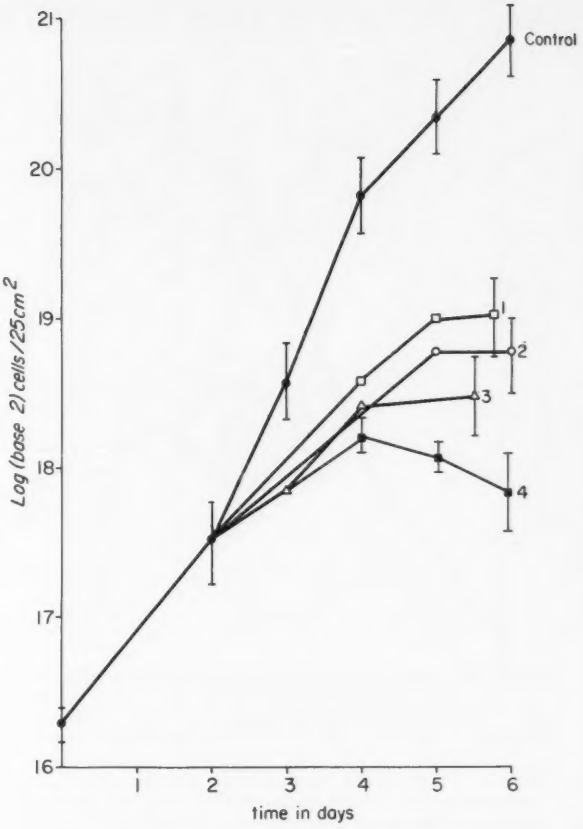

FIGURE 2.—Cell division in the presence of TPL pretreated media. Cells were grown in Eagle's minimum essential medium (MEM). On Day 2, cells were fed with: (1) L-tyrosine-free Eagle's MEM, or (2) Eagle's MEM treated with 0.1 IU/ml of TPL for 15 mins at 37°C followed by ultrafiltration, or (3) L-tyrosine-free Eagle's MEM treated with enzyme as in (2), or (4) Eagle's MEM containing 0.1 IU/ml of TPL (not ultrafiltered). Ultrafiltration was carried out using an Amicon PM-10 membrane under N_2 (17 psig). The dialyzed fetal calf serum component of the media was added after ultrafiltration.

4. L-Tyrosine in treated cultures reached 3% of untreated controls within the first 16 hours of treatment; thereafter, it remained at a low level for at least 28 hours.

Metabolic Responses of Melanoma and Fibroblast Cultures to L-Tyrosine Phenol-lyase

The above data suggest that TPL induces a quiescent, nonproliferative state in melanocytes grown in vitro and in vivo. It therefore seemed of interest to compare this response to that obtained by other growth-blocking agents described by Pardee (10). Figure 5 shows the uptake (and incorporation) of thymidine, uridine, and L-leucine in response to the presence of these blocking agents and to TPL. Only in cultures containing low concentrations of serum was incorporation of precursors into macromolecules not significantly inhibited. Whereas the effect of TPL on L-leucine and thymidine uptake was similar to that observed with the other inhibitors tested, the uptake of uridine in TPL-treated cultures was markedly inhibited. These findings led us to test human fibroblasts under identical conditions (fig 6). These cells, in low passage and of normal origin, generally were more sensitive to the presence of the inhibitors than the melanocytes. As in the response of the melanocytes, the most significant change caused by TPL on treated fibroblasts was a dramatic decrease in the uptake and incorporation of uridine.

In all cultures, with the exception of those treated with hydroxyurea or colchicine, DNA synthesis began 6–12 hours after feeding complete media to the washed but

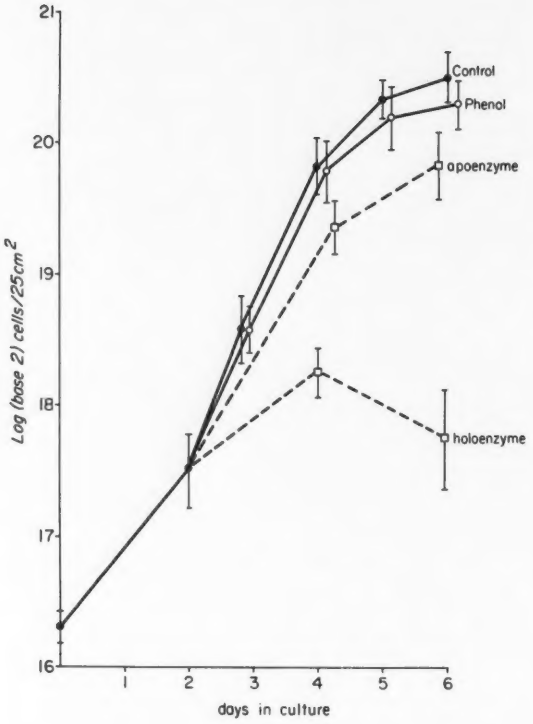

FIGURE 3.—Activity of TPL apoenzyme and holoenzyme and phenol on B16 melanoma growth. Cells were grown in medium 199. Phenol was added to the indicated cultures to give a final concentration of 42 μg/ml. TPL apoenzyme and holoenzyme were added to give a concentration of 0.1 IU/ml. Cultures were treated on Days 2 and 4.

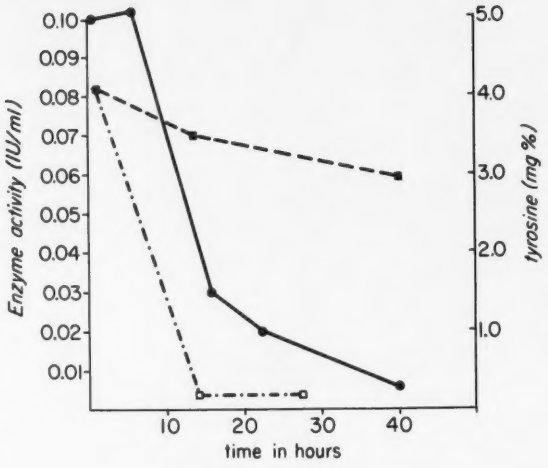

FIGURE 4.—TPL activity and L-tyrosine levels in melanoma culture media. Cells were grown in Eagle's MEM. ——— = TPL activity (IU/ml); ------ = L-tyrosine levels in untreated cultures; –·–·– = L-tyrosine levels in treated cultures.

previously treated cells (figs 7 and 8). Hydroxyurea and colchicine, under the conditions used, irreversibly inhibited proliferation with respect to DNA, RNA, and protein synthesis. There appeared to be no important differences, therefore, in the manner in which TPL affected incorporation of the precursors into fibroblasts or melanocytes or in the patterns of escape upon removal of the inhibitors. B16 mouse melanoma cells were blocked from dividing with TPL for 48 hours and the cells were then subjected to cell cycle analysis as described in the Methods and Materials section. The fraction of cells in G_1, S, and G_2 + M was determined from cellular DNA distributions by a computer-fit program. The normal distribution in untreated melanocytes was 45% in G_1, 37% in S, and 18% in G_2 + M. This distribution was altered appreciably by treatment with TPL for 48 hours. The cells accumulated in G_1, with a distribution of 73% in G_1, 27% in S, and 0 in G_2 + M.

DISCUSSION

Growth of B16 melanoma has been shown to be inhibited by depletion of L-tyrosine and L-phenylalanine. Inhibition of growth has been reported when this deficiency is achieved by dietary restriction (1–4) or by short-term administration of specific degradative enzymes, ie, L-phenylalanine ammonia-lyase (13) and TPL (6,13). The data in the present report indicate that TPL essentially exerts a cytostatic effect on B16 melanoma. Pronounced tumor regression was not observed with a 6-day treatment protocol on established palpable tumors. Proliferation of the melanocytes both in vivo and in vitro is inhibited by the presence of the enzyme. Removal of the enzyme (in vitro) or cessation of treatment (in vivo) resulted in renewed growth. The melanocyte, therefore, as most normal cells, can be viewed as having alternate states of existence, either proliferative or quiescent. L-Tyrosine-restriction, achieved by means of TPL administration, essentially resulted in a nonlethal, quiescent state, characterized by a shutdown of thymidine, uridine, and L-leucine uptake and accumulation of cells in G_1 (or G_0). DNA synthesis began upon stimulation of the cultures by addition of media containing L-tyrosine. These findings are consistent with other reports showing that a variety of suboptimal nutritional conditions result in a block in DNA synthesis and in initiation of a nonproliferative state (14–17).

Pardee (10) showed evidence that normal cells which

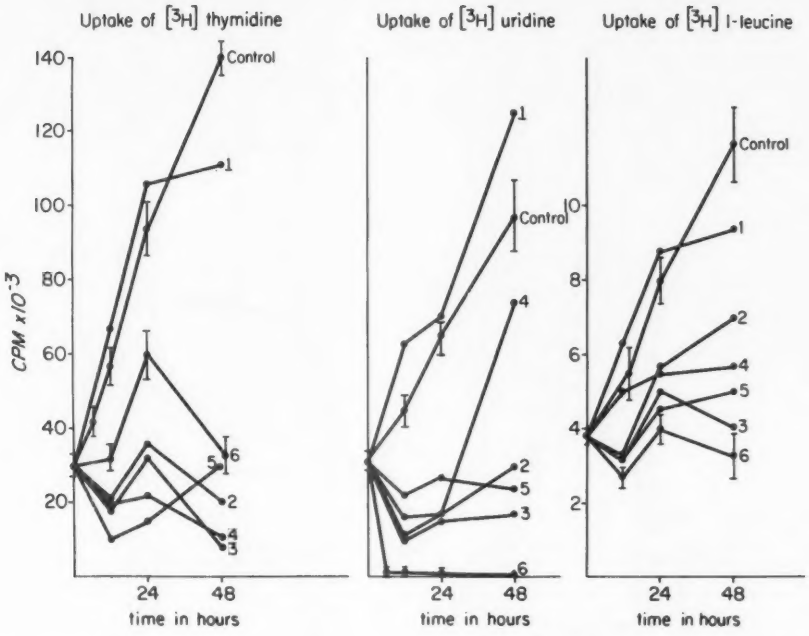

FIGURE 5.—Metabolic response of cultured mouse melanoma cells to proliferative inhibiting conditions including treatment with TPL. Cultures were grown in Eagle's MEM and treated at time 0 by addition of fresh media containing: (1) low serum (0.25% fetal calf serum) + 0.1 mM L-ornithine +0.1 mM hypoxanthine, (2) 1 mM dibutyryl cAMP + 2 mM theophylline, (3) 0.05 mM papaverine, (4) 1 mM hydroxyurea, (5) 10 mg/ml of colchicine, and (6) 0.1 IU/ml of TPL. Cells were labeled for 3 hrs with the precursors at the indicated times and the uptake and incorporation of label were determined.

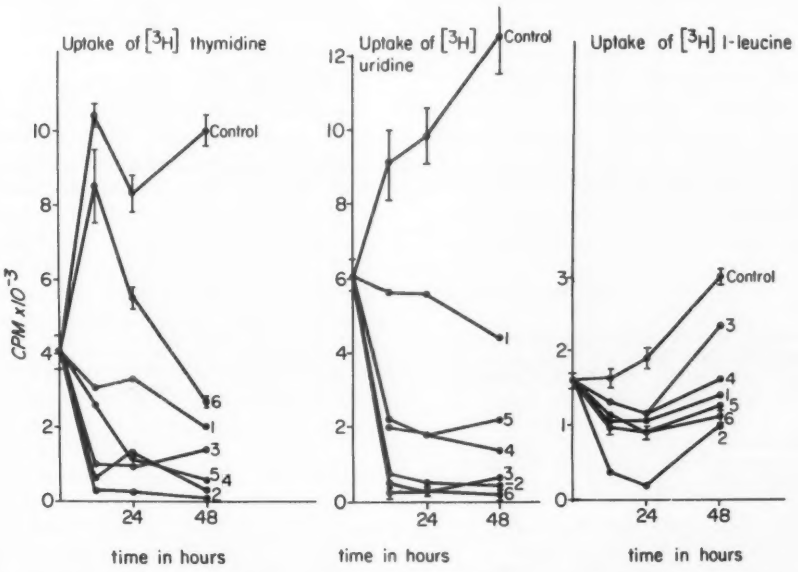

FIGURE 6.—Metabolic response of cultured human fibroblast cells to proliferative inhibiting conditions including treatment with TPL. Fibroblasts were grown, treated, and evaluated as described in fig 5.

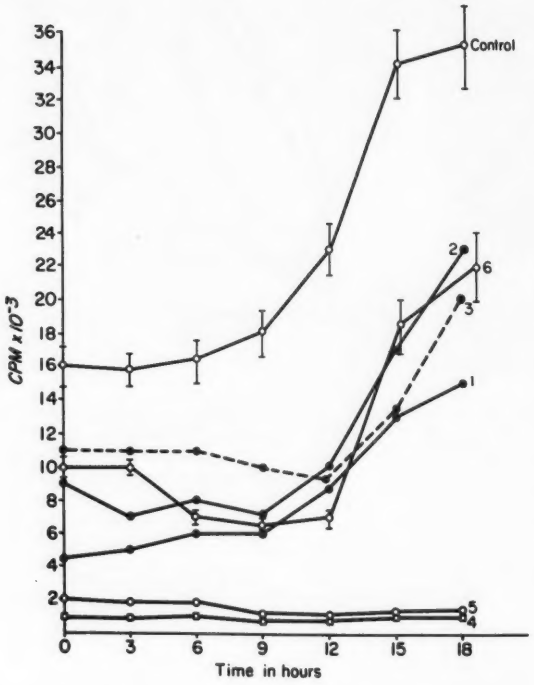

FIGURE 7.—Thymidine uptake of cultured melanoma cells after escape from cell proliferation inhibitors. Cultures treated for 48 hrs as described in fig 5 were released from the inhibiting conditions by rinsing cells twice with Eagle's MEM followed by addition of fresh media. Thymidine uptake was monitored as described in fig 5.

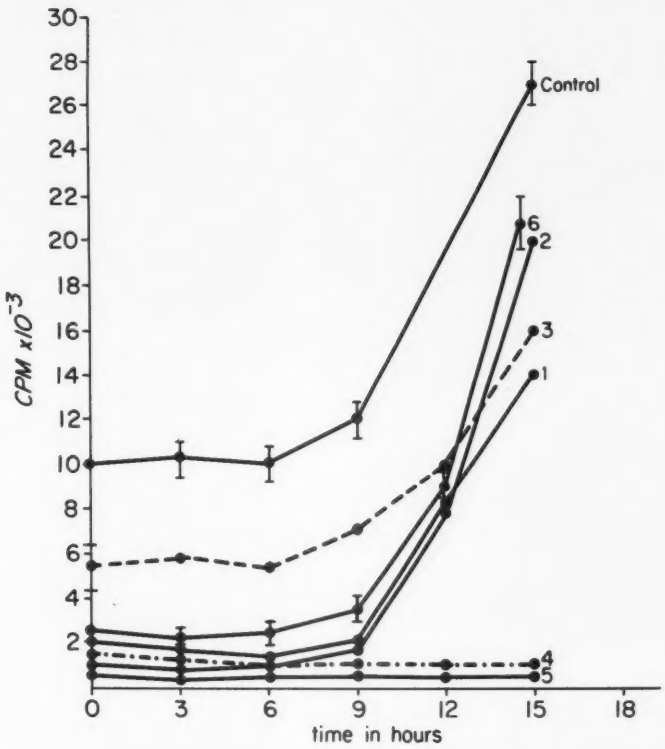

FIGURE 8.—Thymidine uptake of cultured human fibroblasts after escape from cell proliferation inhibitors. Cultures treated for 48 hrs as described in fig 6 were released from the inhibiting conditions by rinsing cells twice with Eagle's MEM followed by addition of fresh media. Thymidine uptake was monitored as described in fig 5.

have reached quiescence by a variety of means are at a "restriction point" that regulates the re-entry of a cell into a new round of the cell cycle when stimulated. It has been suggested (10) that certain tumor cells have lost this mechanism and under suboptimal nutritional conditions tend to arrest randomly in the cell cycle. B16 melanoma cells, however, were capable of arresting in G_1 when depleted of L-tyrosine using TPL and both the melanoma and normal fibroblast cell lines showed a 10–12-hour lag phase before initiating DNA synthesis after removal of blocking agents. Except for the observation that the tumor cells were more resistant to growth blockade, the metabolic response of the normal fibroblasts and the melanoma cells to addition and removal of various blocking agents was similar.

The most pronounced effect in TPL-treated cultures was the near-complete inhibition of uridine uptake. The mechanism for the observed effects on RNA synthesis is not known. Important to our findings, however, is the report of Nakishima and co-workers (17) that L-asparaginase totally abolishes the capability to make both mRNA and rRNA. Kuwano et al (18) found potent control of RNA metabolism via L-asparaginase treatment to occur regardless of whether an L-asparagine-resistant or -sensitive L5178Y cell strain was used. It has also long been known that, in bacterial systems, amino acid limitation precipitates adjustment of cellular activities with blocked RNA synthesis as a principal feature (19).

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Biochemical Properties and Immunogenicity of L-Phenylalanine Ammonia-Lyase: Effects on Tumor-Bearing Mice [1]

Rong-sen Shen, Richard R. Fritz, and Creed W. Abell [2,*]

SUMMARY

L-Phenylalanine ammonia-lyase (PAL) from yeast was used to deplete plasma L-phenylalanine and L-tyrosine in an attempt to achieve inhibition of tumor growth in mice. Plasma L-phenylalanine and L-tyrosine were reduced to nondetectable levels when circulating PAL activity was maintained at ≥ 0.06 unit/ml. Repeated administration resulted in the appearance of anti-PAL antibodies. A radioimmunoassay based on the method of Farr was developed to determine quantitatively the presence of anti-PAL. Sublethal total-body irradiation temporarily suppressed the immunologic response of the host. Long-term specific immunosuppression to PAL was achieved with cyclophosphamide (CPA). A single dose of 180 mg/kg of CPA administered ip to mice 24 hours before, simultaneously with, or 24 hours after 100 units/kg of PAL induced tolerance for 450 days (20 injections of enzyme). The plasma half-life of PAL in CPA-treated mice remained essentially the same as that found after a single injection (25 hours), and anti-PAL was not detected by radioimmunoassay. The successful treatment of neoplasia by PAL probably will require specific immunosuppression of the host to repeated injections of the enzyme.

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Manipulation of the cellular environment of the host to obtain preferential inhibition of neoplastic cell growth represents a challenging approach to cancer therapy. Regulation of concentrations of amino acids in a host can be achieved either by dietary restriction (1–10) or by the use of enzymes that deplete specific amino acids (11–26). Dietary control of nonessential amino acids is not feasible because mammalian cells can synthesize these compounds. Since some neoplasms may not have the ability to synthesize adequate amounts of certain nonessential amino acids, the application of enzymes to deplete specifically these nutrients may provide an unfavorable environment for neoplastic cell growth. This has proven to be the case in certain leukemias that are dependent upon plasma concentrations of L-asparagine (27,28), L-glutamine (28,29), L-serine (30), and L-cysteine (11).

While plasma levels of nonessential amino acids may be manipulated only by the use of enzymes (11–16), levels of essential amino acids may also be altered by dietary manipulation in an attempt to achieve inhibition of tumor growth (1–10). Dietary restriction of an essential amino acid is limited because it takes a long time (4–8 weeks) to attain significant reduction in the concentration of plasma amino acids, and the diet is unpalatable. An alternate approach is to restrict the essential amino acid by administration of an enzyme. Several enzymes have been studied for their therapeutic values. These are L-tryptophanase (17), indolyl-3-alkane-α-hydroxylase (18), L-methioninase (19), L-threonine deaminase (20), L-tyrosine phenol-lyase (21,22), and L-phenylalanine ammonia-lyase (PAL) (22–26).

PAL catalyzes the deamination of L-phenylalanine to *trans*-cinnamic acid and the deamination of L-tyrosine to *p*-coumaric acid (fig 1). Treatment of human acute lymphoblastic leukemia and murine L5178Y lymphoblasts in vitro with PAL resulted in rapid inhibition of cell growth (23,24). Neither the products of L-phenylalanine and L-tyrosine deamination nor inactivated PAL had any effect upon these cells. Administration of PAL at a dose of 100 units/kg/8 hours for 15 doses to BDF_1 mice with L5178Y

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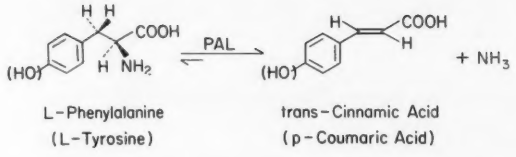

FIGURE 1.—Deamination of L-phenylalanine and L-tyrosine by yeast PAL.

lymphoblastic leukemia rapidly lowered plasma L-phenylalanine and L-tyrosine concentrations, inhibited tumor growth, and produced cures in about 40% of the mice (25). PAL was also evaluated against B16 melanoma maintained in BDF_1 mice by Elmer et al (22). PAL at a dose of 100 units/kg/12 hours for eight doses effectively reduced tumor volume (treated/control value at 4 days of treatment was 0.17), and no toxicity was observed in the treated mice. When partially purified PAL (specific activity, 0.9 unit/mg) was used for the treatment of mice bearing the ascites form of L5178Y and EARAD-1 leukemia, Roberts et al (26) found no significant increase in median survival time.

PAL presumably exerts its therapeutic effect by depriving rapidly dividing neoplastic cells of the essential amino acid, L-phenylalanine. Deprivation of plasma L-phenylalanine may also affect the rate of protein synthesis in and integrity of normal cells. Mammals can utilize phenylpyruvate as a substitute for L-phenylalanine through transamination (31), whereas some neoplastic cells cannot (32). Consequently, by depleting plasma L-phenylalanine enzymatically in conjunction with dietary supplementation of phenylpyruvate, it may be possible to inhibit effectively tumor growth without damaging normal tissues.

PAL has been found in most plants (33,34) and in some fungi and yeasts (35–39) but has not been observed in mammalian or bacterial cells. This enzyme is induced in the yeast *Rhodotorula glutinis* by culture in a medium containing 0.1% L-phenylalanine. Maximal enzyme activity (2 units/g of wet weight of yeast) is reached between mid-log and late-log phase. The enzyme is apparently degraded by proteolysis as the cells enter the stationary phase (39). A highly purified enzyme has been obtained by fractionation with ammonium sulfate and sodium citrate followed by chromatography on DEAE-cellulose and Sephadex G-200 (39). The overall yield is usually about 23%, and the specific activity of the enzyme preparations varies between 2.0 and 3.0 units/mg of protein. The enzyme, prepared as described, yields one major band on standard polyacrylamide gels, on sodium dodecyl sulfate gels, and by isoelectric focusing. Amino acid sequence analysis indicated that the purity of these enzyme preparations is > 90%.

Table 1 summarizes some of the properties of PAL.

TABLE 1.—Properties of PAL (EC 4.3.1.5) from *R. glutinis*

Properties			Ref No.
Equilibrium constant: reaction is essentially irreversible			34
Substrate specificity:	*km*	V_{max}	42
L-Phenylalanine	2.5×10^{-4}M	1.4 μmols/min/mg	
L-Tyrosine	1.5×10^{-4}M	0.7 μmol/min/mg	
Competitive inhibitors:	*ki*		42
D-Phenylalanine	3.3×10^{-3}M		
trans-Cinnamic acid	2.6×10^{-5}M		
p-Coumaric acid	1.8×10^{-5}M		
Molecular weight: 330,000			44
Subunits: 4			33
Active site contains dehydroalanine			42
Isoelectric point: 5.4			39
Optimal pH: 8.75			43
Thermal stability: no loss of activity at 50°C for 10 mins			43

Yeast PAL catalyzes the anti-elimination of the amino group on carbon 2 and the pro-S hydrogen on carbon 3 of L-phenylalanine to yield *trans*-cinnamate (40,41). The enzyme from yeast and from other sources also catalyzes the deamination of L-tyrosine to yield *p*-coumarate; however, PAL from potato tubers and other plants is inactive toward L-tyrosine (41). The Michaelis constants of the yeast enzyme for L-phenylalanine and L-tyrosine are 2.5 and 1.5×10^{-4} M with a V_{max} of 1.4 and 0.7 μmols/minute/mg, respectively (42). PAL has virtually no catalytic activity on any of the common amino acids except L-phenylalanine and L-tyrosine or on the D-isomers or structurally related derivatives of L-phenylalanine and L-tyrosine (42,43). PAL has a molecular weight of about 330,000 and consists of four subunits that are apparently identical (44). The active site of the enzyme contains a dehydroalanine moiety that combines covalently with the α-amino group of the substrate during the catalytic reaction (40,42). The reaction sequence is "ordered uni-bi" with cinnamate (or coumarate) released before ammonium ions (40).

PAL injected ip has a half-life of between 20 and 24 hours in mice, rats, and squirrel monkeys (table 2). The slow clearance of PAL and its stability under physiologic conditions result in effective reduction of circulating L-phenylalanine and L-tyrosine to minimal levels. Circulating PAL activity and plasma levels of L-phenylalanine and L-tyrosine in mice are inversely related; thus, plasma L-phenylalanine is depleted to nondetectable levels when the activity of PAL is maintained at > 0.03 unit/ml of plasma. L-Tyrosine can be depleted by maintaining PAL at > 0.06 unit/ml of plasma. Thus, sufficient PAL activity in the plasma of mice can be obtained (initially, 0.2 unit/ml of plasma) by ip injection of 10 units/kg of body weight to achieve minimal circulating levels of both L-phenylalanine and L-tyrosine (45).

TABLE 2.—Plasma half-life of PAL after single ip injection in different mammals

Species	Specific activity (units/mg)	Dose (units/kg)	Half-life (hrs)	Ref No.
Squirrel monkeys	2.0	100	20	39
Sprague-Dawley male rats	2.0	100	20	39
BDF_1 male mice	1.5–2.0	100	19–24	39, 45
BDF_1 female mice	1.0	60	24	22
BDF_1 female mice + LDH virus	1.0	60	24	22
BDF_1 male mice + L5178Y lymphoblasts	1.5–2.0	100	17–20	45
BDF_1 mice + L5178Y lymphoblasts	0.9	300	30	26
C57BL female mice	1.5–2.0	100	21–24	39, 45
C57BL female mice + B16 melanoma	1.5–2.0	100	18–21	39, 45
C57BL female mice + BW10232 adenocarcinoma	1.5–2.0	100	19–27	39, 45
A/J female mice	1.8	100	23	45
A/J female mice + 15091A anaplastic carcinoma	1.8	100	21	45

Although PAL has no enzymatic activity toward the common amino acids other than L-phenylalanine and L-tyrosine, plasma levels of L-threonine, L-serine, L-methionine, L-isoleucine, and L-leucine in squirrel monkeys were also decreased within 8 hours after administration of 100 units/kg of body weight (46). Plasma levels of all these amino acids returned to normal ranges 24 hours later, whereas L-phenylalanine and L-tyrosine levels remained low. Interestingly, concentrations of L-threonine, L-serine, L-methionine, L-isoleucine, L-leucine, and L-tryptophan increased, whereas concentrations of L-tyrosine and L-phenylalanine decreased in rat brains 4 hours after administration of 100 units/kg of body weight of PAL (46). Presumably, the decrease in plasma levels of some of the large neutral amino acids after PAL administration is due to competition between those amino acids that share the same transport sites across the blood-brain barrier. These fluctuations of brain amino acid levels and concentrations of related biogenic amines resulted in significant alterations of sleep patterns and behavior in squirrel monkeys (46).

Like other microbial enzymes, repeated administration of yeast PAL provokes anti-PAL synthesis in the host, resulting in a marked reduction of the biologic half-life of the enzyme. The presence of antibody in mice was demonstrated by a radioimmunoassay based on a modification of the method of Farr (47,48), although initial attempts to detect anti-PAL using the Ouchterlony double diffusion technique were negative (39). The method is represented in the following diagram:

$$\text{PAL}^* + \text{anti-PAL} \rightleftharpoons \text{PAL}^*\text{:anti-PAL}$$

$$\text{PAL}^*\text{:anti-PAL} + \text{RAMG} \rightleftharpoons \text{PAL}^*\text{:anti-PAL:RAMG}$$

where PAL* is ^{125}I-PAL and RAMG is rabbit anti-mouse globulin. PAL was iodinated with ^{125}I by a modified chloroamine-T oxidation method of Hunter and Greenwood (49), purified on Sephadex G-200, and incubated with mouse sera containing anti-PAL. Iodinated PAL bound to anti-PAL forms a complex that is precipitated by the addition of rabbit anti-mouse antibody, permitting separation of the PAL:anti-PAL complex from free ^{125}I-PAL.

In order to maintain plasma levels of the enzyme, immunologic manipulation of the host or chemical modification of the enzyme appears to be necessary. Modification of PAL by acylation or glycosylation has been tried in this laboratory without success (unpublished data). Chemical modification of PAL often results in a loss in enzymatic activity or stability in biologic fluids. Alternatively, immunologic manipulation of the host with immunosuppressive agents such as radiation or cyclophosphamide (CPA) was found to reduce the accelerated rate of clearance of PAL from the plasma of mice.

The half-life of PAL in the plasma (20 hours) of irradiated mice (400 rads) remained essentially constant after repeated injections (100 units/kg/48 hours for five doses), whereas the same treatment reduced the half-life of the enzyme to about 1 hour in unirradiated controls (50). After 11 injections in 63 days, however, the half-life of the enzyme in the plasma of irradiated mice was reduced to 5 hours. The decrease in enzyme half-life was related to an increase in antibody titer in both unirradiated and irradiated mice. The antibody produced in unirradiated mice was first detected 10 days after the initial injection of enzyme, whereas in irradiated mice, the antibody did not appear until about 27 days after the initial injection of enzyme. Titers of antibody in irradiated mice did not reach those observed in unirradiated mice within 84 days. Furthermore, the initial enzyme activity in the plasma of irradiated mice remained at relatively constant levels through 11 injections (63 days), whereas in unirradiated mice, the enzyme activity decreased after five injections (9 days) to $< 10\%$ of that obtained after a single injection. This study demonstrated that sublethal total-body irra-

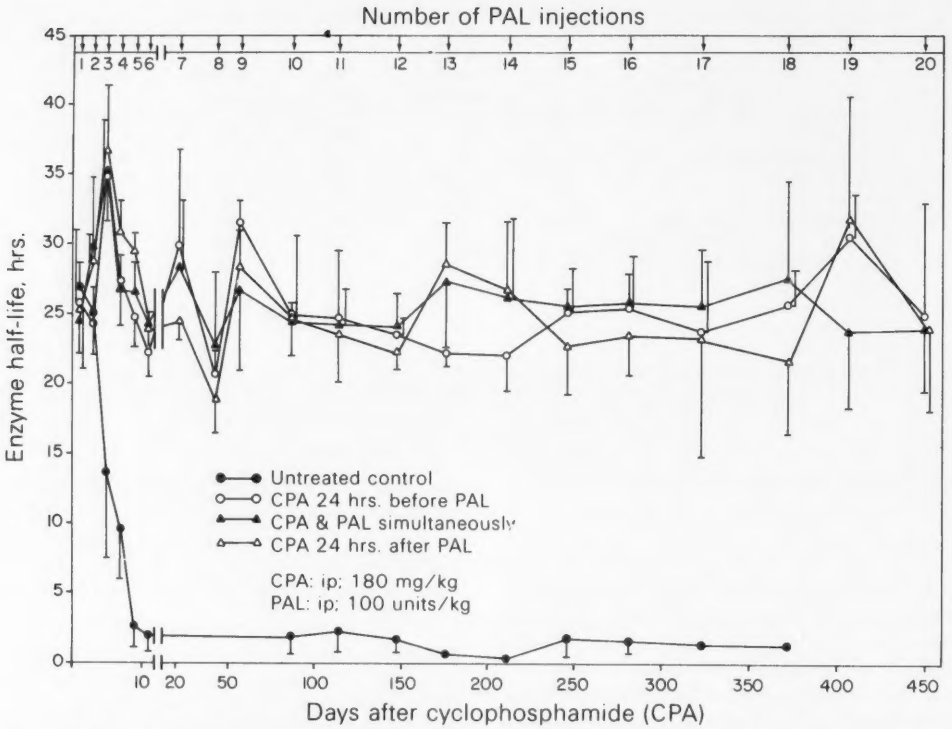

FIGURE 2.—Plasma half-life of yeast PAL in immunosuppressed mice after repeated injections of the enzyme. Groups of mice (C57BL female, 4/group) were treated with 180 mg/kg ip of CPA 24 hrs before, simultaneously with, and 24 hrs after the first dose of 100 units/kg ip of PAL. Subsequent administrations of the enzyme were given at every-other-day intervals (2nd to 6th injections) or intermittently (7th to 20th injections). Untreated control received the same PAL treatments but without CPA. Blood was collected from the tail vein at various time points after PAL administration, plasma was prepared, enzyme activity was determined, and the half-life was calculated as previously described (45). Each point and bar represents the mean and SD of 4 mice, except for the last 2 points of the untreated control which represent an average of 2 mice.

diation may be used to delay, for a limited period of time, the accelerated rate of clearance of PAL from the plasma of normal and tumor-bearing mice.

The disadvantage of radiation as a means of immunosuppression is that the unresponsiveness achieved is nonspecific and only temporary. Consequently, cytotoxic drugs were tested in an attempt to suppress specifically humoral immunity against PAL while permitting other immune responses to remain intact. Moreover, the use of immunosuppressive drugs may enhance the therapeutic effectiveness of PAL because they exhibit antineoplastic activity. CPA, an alkylating agent, has been shown to have antineoplastic and immunosuppressive activities (51). Consequently, this agent was administered in combination with PAL (figs 2 and 3). Immunosuppression for > 450 days was achieved when a single dose of CPA of 180 mg/kg was administered to C57BL mice 24 hours before, simultaneously with, or 24 hours after 100 units/kg of PAL. A total of 20 PAL injections have been given to each mouse and the half-lives of PAL in the plasma of CPA-treated mice remained essentially the same as those found after a single injection (25 hours). The presence of antibodies against PAL was not detected by radioimmunoassay. In contrast, the half-life of PAL in the plasma of mice not treated with CPA was reduced to 2 hours after six injections of PAL in 11 days and remained at approximately 1.5 hours thereafter. The reduction in enzyme half-life was accompanied by an increase in titer (several thousand fold) of antibodies against PAL (fig 3). Interestingly, all mice in this group died within 380 days after the initiation of the experiment.

The successful treatment of neoplasia by PAL or other enzymes probably will necessitate the development of methods for specific immunosuppression of the host to repeated injections of the enzyme. Alternatively, enzymes can be chemically modified prior to their administration to reduce their immunogenicity. Treatment of mouse melanomas and mammary tumors with CPA and PAL and human acute lymphoblastic leukemia with CPA and *Escherichia coli* L-asparaginase is currently under investigation. Initial clinical studies with CPA at a dose

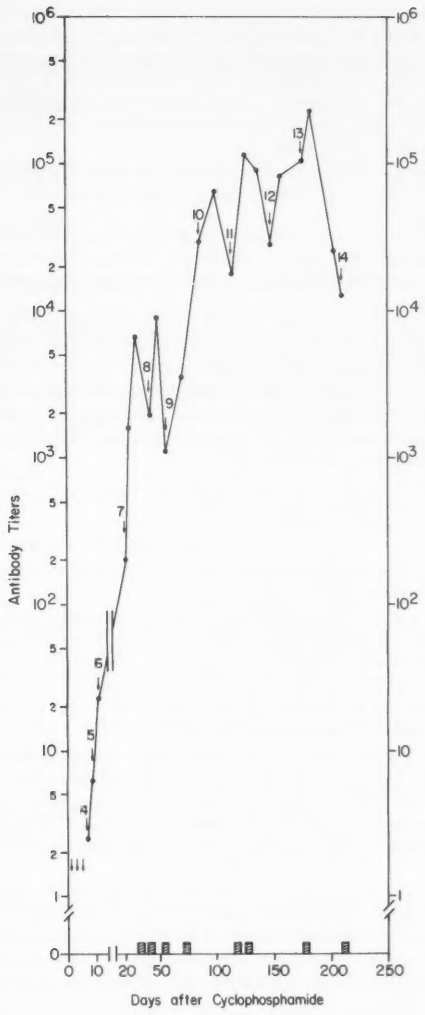

FIGURE 3.—Antibody titers to PAL in plasma of mice after repeated enzyme injections. Female C57BL mice, with and without CPA treatment, were given 20 doses of PAL (100 units/kg, ip) intermittently over a period of 450 days. Plasma half-life of the enzyme after each injection is presented in fig 2. Blood was collected from the tail vein prior to and several days after each enzyme injection, plasma was prepared, and antibody against PAL was determined by radioimmunoassay (*see* text). Anti-PAL in plasma of untreated controls was first detected 11 days after the initial administration of the enzyme. The titers increased several thousand fold thereafter following repeated enzyme injections. Antibodies against PAL were not detected in any group of the CPA-treated mice (boxes with diagonal lines). Each point represents an average of 4 mice. Arrows with Nos. indicate the time and No. of enzyme injections.

of 1000 mg/m^2 and L-asparaginase at a dose of 10,000 units/m^2/week for three doses have proved to be successful in achieving a 1-year remission in a 21-year-old male with acute lymphoblastic leukemia who had become resistant to Adriamycin, cytosine arabinoside, vincristine, and prednisone. The induction of long-term immunosuppression to L-asparaginase may provide improved therapy for those patients who develop resistance to the known useful therapeutic drugs.

In conclusion, PAL exhibits the following criteria for enzyme therapy: (*a*) the reaction catalyzed is essentially irreversible; (*b*) no exogenous co-factor is required; (*c*) the enzyme is active and highly stable in vivo; (*d*) the enzyme has substrate specificity for only L-phenylalanine and L-tyrosine; (*e*) the enzyme has a long half-life in the plasma of animals; and (*f*) a nonpathogenic organism provides a readily available source. The two major limitations of the therapeutic application of PAL, however, are the host's immunologic response to PAL and the difficulty of obtaining preferential inhibition of neoplastic cell growth. Specific immunosuppression to repeated administration of PAL for > 1 year has been successfully achieved. Thus, low levels of plasma L-phenylalanine may be controlled for desired time periods. Current studies are designed to evaluate PAL in combination with potential therapeutic drugs or analogs that share with L-phenylalanine the same mode of cellular transport.

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Tumor Therapy by Deprivation of L-Methionine: Rationale and Results[1]

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SUMMARY

Published reports indicate that normal rodent cells can grow in medium containing either L-methionine or L-homocysteine, whereas malignant rodent cells have an absolute requirement for L-methionine. Our studies with two normal human cell lines (fetal lung fibroblasts and bladder epithelial cells) exhibit equal growth in media containing either L-methionine or L-homocysteine. The same is true for five malignant human cell lines (carcinoma of the cervix [HeLa], adenocarcinoma of the breast [AlAb], acute lymphoblastic leukemia [MOLT-3], Wilms' tumor [SK-NEP-1], and reticulum cell sarcoma [T-77]), whereas four other malignant cell lines (adenocarcinoma of the breast [SK-BR-2-III], the two lymphoblastic leukemias [CCRF-HSB-2 and CCRF-SB], and a neuroblastoma [SK-N-MC]) have absolute requirements for L-methionine. Two malignant cell lines, an adenocarcinoma of the lung (A549) and an adenocarcinoma of the pancreas (Capan-1), showed restricted growth under the experimental conditions used. L-Methioninase (L-methionine-α-deamino-γ-mercaptomethane-lyase, EC 4.4.1.11) at a concentration of 0.1 unit/ml leads to complete growth inhibition of cell cultures of both the normal human fetal lung fibroblasts (F-136-35-56) and the acute lymphoblastic leukemia (CCRF-HSB-2). L-Homocysteine-thiolactone in medium containing L-methioninase could partly "rescue" the normal but not the malignant cells.

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L-Methionine, an amino acid constituent of most, but not all, proteins, is probably the most important organic sulfur-containing compound; it is essential in the initiation of polypeptide chains and is the chief methyl donor for the methylation of DNA, RNA, protein, and other biologic substrates. In the last few years, a number of reports have dealt with the varying capabilities of mammalian cells, both benign and malignant, to grow in tissue culture media containing L-homocysteine[4] instead of L-methionine (1–6). Thus, a survey of six such studies, as shown in table 1, reveals that all seven normal cell lines derived from normal tissue, both rodent and human, show equal growth in medium containing either L-methionine or L-homocysteine.

In contrast, the malignant cell lines behaved uniformly only in the rodent series, with all three cell lines having an absolute requirement for L-methionine, whereas the two human malignant cell lines showed differing behavior. Two virus-transformed human skin fibroblasts also exhibited an absolute requirement for L-methionine.

Thus, the emerging picture is clear-cut only in the rodent cell lines, where normal cells grow well in L-methionine- or L-homocysteine-containing medium, and the malignant cells have an absolute requirement for L-me-

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[4]DL-Homocysteine was used in certain of these studies, but it is likely that the L-stereoisomer is the responsible molecule.

TABLE 1.—Growth requirements of mammalian cells for L-methionine and replacement by L-homocysteine

	No. of cell lines tested	No. of cell lines with—		Ref No.
		Equal growth in L-methionine- or L-homocysteine-containing medium	Absolute requirement for L-methionine	
Normal cells				
Rodent	3	3	0	1–3
Human	4	4	0	2,4
Malignant cells				
Rodent	3	0	3	2,4,5
Human				
Malignant	2	1	1	2,6
Virus-transformed	2	0	2	4

thionine. In the small series of human cell lines, the response to L-homocysteine-containing medium is equivocal, whereas the two virus-transformed cells behave like the experimental malignant cells.

In order to extend these very limited reports on L-methionine requirements, a larger series of experimental, and especially human, cell lines were investigated for their requirements for this amino acid. The present report is an updated compilation of our results with human cell lines, and provides data concerning the therapeutic exploitation of observed differences of L-methionine requirements using an L-methionine-degrading enzyme (L-methioninase: L-methionine-α-deamino-γ-mercaptomethane-lyase, EC 4.4.1.11). Initial screening data for L-methionine requirements were published elsewhere (7).

MATERIALS AND METHODS

Cell Lines

The human cell lines F-136-35-56 (fetal lung fibroblasts), HCV-29 (bladder epithelial cells) (8), AlAb and SK-BR-2-III (adenocarcinomas of the breast) (9,10), SK-N-MC (neuroblastoma) (11), A549 (adenocarcinoma of the lung) (12), Capan-1 (adenocarcinoma of the pancreas) (13), MOLT-3 (acute lymphoblastic leukemia, a T-cell neoplasm) (14), SK-NEP-1 (Wilms' tumor) (8), and T-77 (reticulum cell sarcoma) (13) were kindly supplied by Dr. Jørgen Fogh. HeLa (cervical carcinoma), CCRF-HSB-2 and CCRF-SB (two human acute lymphoblastic leukemias, T- and B-cell neoplasms, respectively), and Walker 256 (rat carcinoma) were obtained from the American Type Culture Collection, Rockville, Md. All cells were regularly tested for the presence of mycoplasma according to the method of Chen (15) and were found to be negative.

L-Methioninase was extracted from *Clostridium sporogenes* according to the procedure reported earlier (16,17). For the enzyme studies, the middle fractions of the enzyme-active peak derived from the Sephadex G200 gel filtration were used.

Culture Techniques

Cells were carried in McCoy's 5A medium (18) as outlined elsewhere (7). All experiments were carried out in triplicate, and the rate of growth was assessed by determination of total protein (19). L-Methioninase was filter-sterilized using 0.45-μm Millipore filters (Millipore Corp, Bedford, Mass).

RESULTS AND DISCUSSION

Table 2 is a summary of our studies with two normal and 11 malignant human cell lines. Whereas the two normal cell lines, namely, the fetal lung fibroblasts (F-136-35-56) and the human epithelial bladder cells (HCV-29), showed equal growth in medium containing either L-methionine or DL-homocysteine, a wide variety was observed in the malignant cells for their capacity to grow in these two media. Adenocarcinoma of the breast (SK-BR-2-III), the two acute lymphoblastic leukemias CCRF-HSB-2 (T cells) and CCRF-SB (B cells), and a neuroblastoma (SK-N-MC) showed no growth at all in the DL-homocysteine-containing medium. An adenocarcinoma of the lung (A549) and an adenocarcinoma of the pancreas (Capan-1) exhibited restricted growth in medium containing DL-homocysteine instead of L-methionine, whereas an adenocarcinoma of the breast (AlAb), HeLa (carcinoma of the cervix), acute lymphoblastic leukemia (MOLT-3, T cells), Wilms' tumor (SK-NEP-1), and a reticulum cell sarcoma (T-77) grew equally well in both L-methionine- and DL-homocysteine-containing media.

Thus, a "general rule" connecting absolute requirements for L-methionine with malignancy and equal growth of cells in L-methionine- or L-homocysteine-containing media with benign cell character, while still a possibility in experimental cell lines, is ruled out completely for

TABLE 2.—Growth requirements of human normal or malignant cells for L-methionine and replacement by DL-homocysteine

Cell line	Designation	Growth	Response
Normal cells			
Fetal lung fibroblasts	F-136-35-56	Monolayer	Equal growth
Bladder, epithelial cells	HCV-29	Monolayer	Equal growth*
Malignant cells			
Adenocarcinoma of the breast	SK-BR-2-III	Cell suspension	Absolute requirement*
Lymphoblastic leukemia	CCRF-HSB-2 (T cells)	Cell suspension	Absolute requirement
Lymphoblastic leukemia	CCRF-SB (B cells)	Cell suspension	Absolute requirement
Neuroblastoma	SK-N-MC	Monolayer	Absolute requirement
Adenocarcinoma of the lung	A549	Monolayer	Restricted growth
Adenocarcinoma of the pancreas	Capan-1	Monolayer	Restricted growth
Adenocarcinoma of the breast	AlAb	Monolayer	Equal growth
Carcinoma of the cervix	HeLa	Monolayer	Equal growth
Acute lymphoblastic leukemia	MOLT-3 (T cells)	Cell suspension	Equal growth
Wilms' tumor	SK-NEP-1	Cell suspension	Equal growth*
Reticulum cell sarcoma	T-77	Cell suspension	Equal growth

*Preliminary results.

human tissues. However, our studies confirmed, for two more normal cell lines, the reports on seven normal cell lines (1–4) whose growth was equal in medium containing either L-methionine or L-homocysteine. Thus, so far, there is no exception to this observation with normal rodent or human cells. Should further studies with a wide variety of normal experimental and human cell lines conform with this data, then, theoretically, a new approach to selectively kill or inhibit the cell growth of certain malignant cells, those with an absolute requirement for L-methionine, would be possible. For protection of the normal cells, L-homocysteine would have to be provided.

The deprivation of L-methionine can easily be achieved with L-methioninase extracted and purified from *C. sporogenes* (16,17). L-Methioninase has the capability of irreversibly degrading L-methionine to α-ketobutyric acid, NH_3, and methanethiol. Indeed, both cell lines studied for growth inhibition (F-136-35-56 and CCRF-HSB-2) were completely inhibited by 0.1 unit/ml of L-methioninase. This is the amount of enzyme necessary for complete breakdown within 1 hour of all the L-methionine available in the modified McCoy's medium. Unfortunately, the specificity of such L-methionine-degrading enzymes is not a narrow one; that is, besides L-methionine, also L-homocystine, L-homocysteine, L-cysteine, and L-cystine are rapidly degraded. This is true not only for the L-methioninase isolated from *C. sporogenes* but also for the L-methioninase extracted and purified by Tanaka et al and Soda et al from *Pseudomonas ovalis* (20,21).

Thus, in order to keep the normal cells alive, one would have to have an enzyme with much higher selectivity for L-methionine, ie, one capable of degrading L-methionine but not L-homocysteine; alternatively, one would have to find ways to provide L-homocysteine in a form which would not be a substrate for the L-methioninase but still would sustain cell growth of normal cells in L-methionine-free medium.

The discovery of L-methionine-degrading enzymes selectively and irreversibly catalyzing the degradation of L-methionine, but not L-homocysteine, might not be an easy task, since all the purified or semipurified L- and D-methioninases reported so far have no exclusive specificity for L-methionine (16,21,22). Indeed, due to the close steric relationship of L-homocysteine to L-methionine, an enzyme with such high selectivity might be hard to find. The second possibility, namely, to circumvent the breakdown of L-homocysteine by using L-homocysteine derivatives, seems to be, at least at this time, more promising. DL-Homocysteine-thiolactone, which can be used instead of L-homocysteine for support of growth of normal adult rat liver fibroblasts (23) and our normal fetal lung fibroblast line (F-136-35-56), is a poor substrate for semipurified *C. sporogenes* L-methioninase; relative values for deamination under standard conditions (16) are 1.0 for L-methionine and 0.03 for DL-homocysteine-thiolactone. As demonstrated in figure 1, initial experiments revealed that both normal fibroblasts (F-136-35-56) and acute leukemic cells (CCRF-HSB-2) were completely inhibited by 0.1 unit of L-methioninase/ml of growth medium, but that only the fibroblasts showed cell growth in medium containing the enzyme and L-homocysteine-thiolactone. While the "rescue" from the L-methioninase treatment is not complete with the fibroblast line, it is a step in the right direction.

Presently, ongoing studies are trying to elucidate this "rescue" mechanism. It could be argued that a rapid inactivation of the enzyme would ultimately allow the thiolactone, after nonenzymatic ring opening and reformation of free L-homocysteine, to act as such in this "rescue" operation. However, the thiolactone in our preliminary

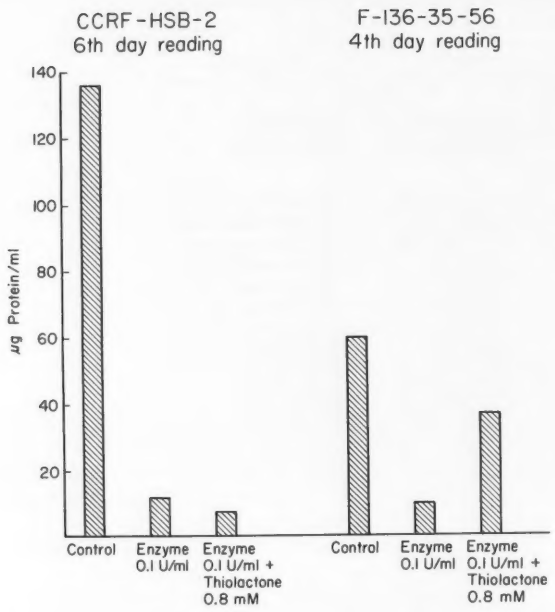

FIGURE 1.—Effect of L-methioninase alone or in combination with L-homocysteine-thiolactone upon the growth of CCRF-HSB-2 and F-136-35-56 in tissue culture. Ordinate = μg of protein/ml of cells in tissue culture.

studies has not shown any evidence of substantial ring opening under the conditions of the experiment.

We intend to perform similar rescue studies in vivo in mice. Should such protection of the host from L-methioninase-induced L-methionine "starvation" be feasible, by co-administration of DL-homocysteine-thiolactone or other L-homocysteine derivatives, then it should also be possible to inhibit the growth of L-methionine-dependent tumors in vivo.

While extensive testing for L-methionine requirements remains to be done (and enzymatic background for both benign and malignant cells has to be provided), the above outline of the rationale and our results provide encouragement and impetus.

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L-Cyst(e)ine Requirements of Malignant Cells and Progress Toward Depletion Therapy[1]

Jack R. Uren* and Herbert Lazarus[2,3]

SUMMARY

The L-cyst(e)ine requirements of normal and malignant cells are reviewed and expanded within the context of establishing whether the measurement of γ-cystathionase levels constitutes a predictive test for tumor sensitivity to L-cyst(e)ine depletion. The ability of both purified L-cysteine desulfhydrase and γ-cystathionase to inhibit the growth of the L-cystine-dependent L1210 leukemia in culture is presented, as well as approaches to circumvent the limitations of these enzymes for in vivo therapy. The ability of propargylglycine to inhibit L-cysteine biosynthesis in vivo is reviewed for its possible use in combination therapy. In addition, the ability of poly D,L-alanine modification of *Escherichia coli* L-asparaginase to increase the plasma half-life in mice tenfold as well as to decrease the immunogenicity of the enzyme is presented.

[Cancer Treat Rep 63:1073–1079, 1979]

L-Cyst(e)ine Requirements of Malignant Cells

A prerequisite for the rational design of a cancer chemotherapeutic agent is the identification of a target substance which is differentially required by normal and malignant tissues. We believe L-cysteine (and/or L-cystine) to be such a target substance. In vivo, L-cysteine is a nonessential amino acid which is biosynthesized by the cystathionine pathway depicted in figure 1. L-Homocysteine, which is derived by demethylation of L-methionine, is condensed with L-serine by cystathionine synthetase, producing cystathionine. Cystathionine is then cleaved to L-cysteine, ammonia, and α-ketobutyrate by γ-cystathionase. Cell culture studies in our laboratories using various normal and malignant established cell lines (1–4) and studies reported by Ohnuma et al (5) using cells derived from patients with acute lymphocytic and myelocytic leukemias in short-term culture have all demonstrated that cells require preformed L-cystine even though adequate levels of L-methionine and L-serine were present. Studies at the Sidney Farber Cancer Institute by Lazarus et al (2), Livingston et al (3), and Iglehart et al (4) (table 1) have demonstrated that, if cystathionine is added to L-cystine-free media, a differential growth response between normal and leukemic cells in culture can be demonstrated. Normal cells can grow in the absence of L-cystine if cystathionine is supplied in the media, whereas leukemic cells cannot. This differential growth requirement was correlated with the level of γ-cystathionase present in the cells (normal cells have a tenfold higher level than leukemic cells).

With solid tumors as well (table 2), Lazarus et al have found a correlation between the cystathionine growth requirement and γ-cystathionase activity among human melanoma and neuroblastoma cells in vitro. Unlike the case for leukemic cells, some solid tumors apparently do not respond to L-cystine depletion in the presence of cystathionine, although they appear to be in the minority. Grossman et al (6) have also demonstrated a reduction in γ-cystathionase levels among six Morris hepatomas, with four of the six being <10% of control liver levels and

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TABLE 1.—Correlation between L-cystine/cystathionine growth requirements and γ-cystathionase activity among various normal and leukemic human and murine continuous cell lines*

Species	Donor	% growth inhibition† L-Cystine-free	% growth inhibition† L-Cystine-free + cystathionine	γ-Cystathionase activity (nmols/mg of protein/hr)
Man	Normal	> 100 (8)‡	14 ± 9 (8)‡	3.5 ± 0.8 (4)‡
Man	Leukemic	> 100 (8)	93 ± 17 (8)	0.4 ± 0.46 (4)
Mouse	Normal	> 100 (3)	3 ± 6 (3)	1.4 ± 0.3 (2)
Mouse	Leukemic	> 100 (5)	> 100 (5)	0.18 ± 0.06 (4)

*Data from refs 3 and 4.

†$100 - \left(\frac{\text{cell No. in deficient medium} - \text{original cell No.}}{\text{cell No. in complete medium} - \text{original cell No.}}\right) \times 100$, after 1–2 days in culture.

‡Group average and SD (No. of cell lines tested).

TABLE 2.—Correlation between L-cystine/cystathionase growth requirements and γ-cystathionase activity among various human continuous solid tumor cell lines

Cell line	Diagnosis	% growth inhibition* L-Cystine-free‡	% growth inhibition* L-Cystine-free + cystathionine‡	γ-Cystathionase activity† (nmols/mg of protein/hr)
NH	Melanoma	100	100	0.17
HPR-1	Melanoma	100	100	0.12
LMP	Melanoma	100	100	0.17
HAJG-1	Melanoma	100	0	0.72
SK-N-MC	Neuroblastoma	100	100	0.22
SK-N-SH	Neuroblastoma	100	100	0.22
IMR	Neuroblastoma	100	50	0.33

*$100 - \left(\frac{\text{cell No. in deficient medium} - \text{original cell No.}}{\text{cell No. in complete medium} - \text{original cell No.}}\right) \times 100$, after 7–14 days in culture.

†^{14}C-Homoserine deaminase assay (16).

‡Medium: minimum essential medium minus L-cystine, supplemented with 10% dialyzed fetal bovine serum + 0.2 mM cystathionine or 0.1 mM L-cystine.

the remaining two between 20% and 60%. Livingston et al (3) (table 3) have measured a fivefold to tenfold decrease in the specific activity of γ-cystathionase concurrent with the development of thymomas induced by Moloney virus or spontaneously in AKR mice. The biochemical properties of the enzyme isolated from leukemic cells were indistinguishable from enzyme isolated from normal cells, and there was no evidence of a dissociable inhibitor (4). Unlike the case of L-asparagine synthetase (7–11), we have found no evidence for γ-cystathionase derepression in animals receiving L-cystine-free diets or in cells cultured in L-cystine-free media. Therefore, we feel that measurements of γ-cystathionase specific activity may be of predictive value in determining which tumors may respond to L-cyst(e)ine-depletion therapy. The differential L-cysteine biosynthetic ability between normal and malignant lymphoid cells may also explain the therapeutic activity of L-selenocystine, which has been shown to inhibit the influx of L-cystine into leukemic cells and to cause tumor regression in experimental animals and man (12–14).

Enzymatic L-Cyst(e)ine Depletion

Among the commonly described enzymes which degrade L-cysteine and/or L-cystine, only L-cysteine desulfhydrase and β- and γ-cystathionases require no additional substrates which might limit their in vivo activity (fig 2). Although all three enzymes do require the coenzyme pyridoxal phosphate which may restrict their activity in vivo, we have found no change in the plasma

TABLE 3.—γ-Cystathionase activity in extracts of normal and tumored mouse thymus*

Mouse strain	Thymus	Age (mos)	γ-Cystathionase activity (nmols/mg of protein/hr)	Group average
Swiss	Normal	1	0.27 ± 0.19	
Swiss	Normal	3	0.18 ± 0.03	
Swiss	Normal	4	0.14	
Swiss	Normal	8	0.15	0.17 ± 0.055
AKR	Normal	1	0.17 ± 0.05	
AKR	Normal	9–10	0.11	
Swiss	MuLV tumor	2–3	0.04 ± 0.02	
Swiss	MuLV tumor	3–4	0.02 ± 0.01	
AKR	Spontaneous tumor	7.5	0.028 ± 0.017	
AKR	Spontaneous tumor	8.5	0.016	0.024 ± 0.011
AKR	Spontaneous tumor	9	0.014 ± 0.006	

*Data from ref 3.

clearance rates of L-cysteine desulfhydrase and γ-cystathionase in mice with and without added pyridoxal phosphate in the assay mixture. Both purified L-cysteine desulfhydrase and γ-cystathionase will inhibit the incorporation of ^{3}H-thymidine into DNA in the L-cystine-dependent L1210 leukemia in vitro (table 4). L-Cysteine desulfhydrase requires the presence of a reducing agent which confirms its substrate specificity and the lack of a toxic contaminant. Human plasma contains about equimolar L-cysteine and L-cystine at a level of about 40 μM (15). We have found *no* information to suggest whether these compounds are in equilibrium in vivo such that the removal of one form will deplete the other. A comparison of the properties of these enzymes is shown in table 5. The two most significant properties are the 1000-fold greater specific activity of the L-cysteine desulfhydrase over the γ-cystathionase and the tenfold longer plasma half-life of the γ-cystathionase over the L-cysteine desulfhydrase. Ip administration of 10 IU of L-cysteine desulfhydrase in mice caused no measurable plasma L-cyst(e)ine depletion, presumably due to its rapid plasma clearance; the administration of 5 IU of γ-cystathionase, on the other hand, caused a transient plasma L-cyst(e)ine depletion to 60% of control levels (16). The low specific activity and limited solubility of this enzyme precluded more extensive therapy.

Inhibitors of L-Cysteine Biosynthesis

The combined use of an inhibitor of amino acid biosynthesis with an amino acid-destroying enzyme offers a possible mode of attack on tumor cells which may be harbored within organs of high amino acid synthetic capacity as well as a decreased amount of enzyme necessary to maintain plasma amino acid depletion. Propargylglycine, an enzyme-activated irreversible inhibitor of γ-cystathionase, has been described by Abeles and Walsh (17) (fig 3). This acetylenic amino acid is thought to undergo an enzyme-catalyzed rearrangement to a reactive ketene structure on the enzyme surface with a subsequent covalent binding to the enzyme. The covalent nature of this inactivation was confirmed in our laboratory by the preparation of the tritiated amino acid analog and sodium

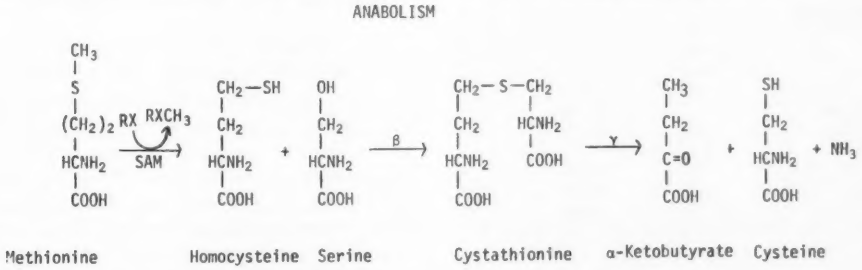

FIGURE 1.—Cystathionine pathway for L-cysteine biosynthesis.

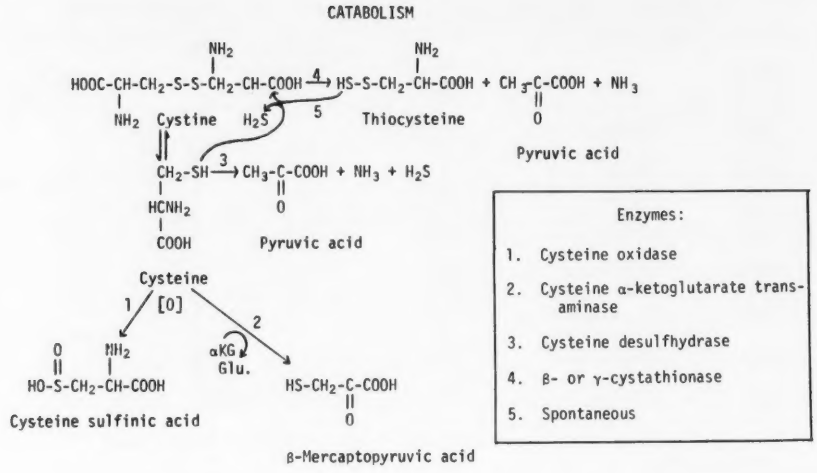

FIGURE 2.—Major enzymatic reactions which catabolize L-cysteine and L-cystine.

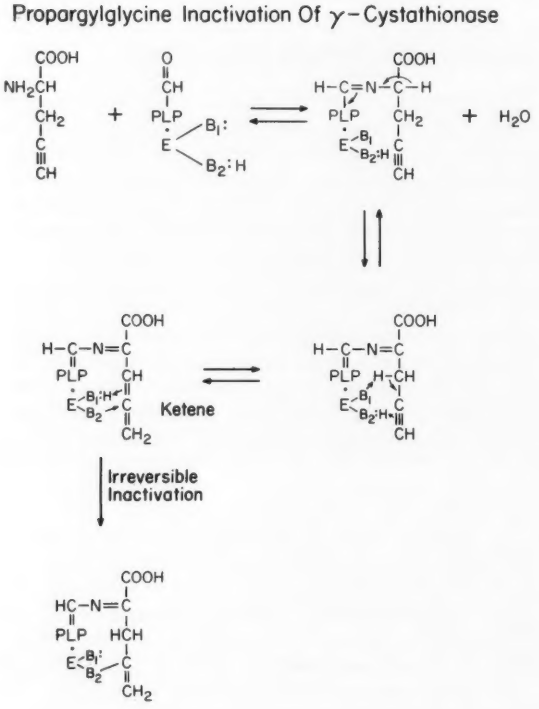

FIGURE 3.—Proposed mechanism for propargylglycine inactivation of γ-cystathionase (17).

dodecylsulfate (SDS) gel electrophoresis of propargylglycine-treated γ-cystathionase before and after SDS denaturation (fig 4). This experiment demonstrates the covalent nature of the inactivation product as well as the necessary presence of a catalytically active enzyme in order to obtain a reaction with the inhibitor. A single injection (14 mg/kg) of propargylglycine completely inactivated mouse liver γ-cystathionase in vivo (fig 5). Activity began to reappear 1 day later, presumably due to synthesis of new enzyme. This procedure would induce both a cystathioninemia and a cystathioninuria in mice. The compound was not toxic to cells in culture or to mice when tested with an adequate source of L-cystine, but was highly toxic in the absence of L-cystine, testifying to its selectivity towards L-cysteine biosynthesis (16).

Effects of Chemical Modification on Enzyme Clearance Rate and Immunogenicity

The rapid plasma clearance of L-cysteine desulfhydrase has precluded therapeutic trials with the enzyme either alone or in combination with propargylglycine. We therefore have examined the effects of chemical modification of *Escherichia coli* L-asparaginase on clearance rate and immunogenicity in the hope of finding a general procedure to alter these very important therapeutic parameters. L-Asparaginase was chosen because of its availability and direct clinical importance. Following the pioneering work of Sela (18), we polymerized long chains of D,L-alanine to the enzyme's lysine residues by the D,L-alanine-*N*-carboxyanhydride procedure. After ammonium

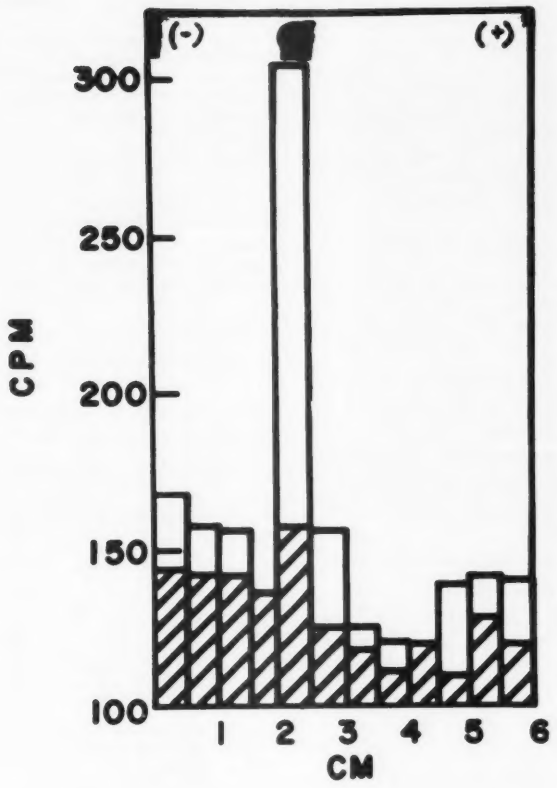

FIGURE 4.—SDS-polyacrylamide gel electrophoresis of γ-cystathionase reacted with tritiated propargylglycine. Shaded bars = enzyme which was denatured prior to reaction with propargylglycine. The top figure presents a Coomassie blue-stained gel (16).

TABLE 4.—Effect of enzymatic L-cyst(e)ine depletion on L1210 leukemia growth in vitro

Enzyme	Source†	Amount (IU/ml)	Without reduction		With reduction*	
			cpm‡	% inhibition	cpm‡	% inhibition
None	—	0	68,000	0	112,300	0
L-Cysteine desulfhydrase	*Salmonella typhimurium*	0.10	64,000	7	5700	95
L-Cysteine desulfhydrase	*Enterobacter cloacae*	0.10	47,400	31	6300	94
None	—	0	30,200	0	—	—
γ-Cystathionase	Rat liver	0.10	11,100	63	—	—
γ-Cystathionase	Rat liver	0.01	22,800	25	—	—

*Presence of 1 mM dithiothreitol.
†Enzymes obtained as in ref 16.
‡Acid-insoluble cpm after a 1-hr pulse with ^{3}H-thymidine following a 24-hr incubation of the cells with the enzyme.

TABLE 5.—Comparative properties of L-cysteine desulfhydrase and γ-cystathionase from various sources*

	Enzyme			
	L-Cysteine desulfhydrase	L-Cysteine desulfhydrase	γ-Cystathionase	γ-Cystathionase
Source	*Enterobacter cloacae*	*Salmonella typhimurium*	Rat liver	*Neurospora*
Substrate	L-Cysteine	L-Cysteine	L-Cystine	L-Cystine
Km	0.3 mM	0.2 mM	0.07 mM	0.03 mM
Specific activity (μmols/min)	114	451	0.28	0.10
Optimum pH	8.5–9.0	8.5–9.0	7–8	7–8
Molecular weight	230,000	230,000	190,000	—
Plasma half-life	10 mins	10 mins	1.7 hrs	—

*Data from ref 16.

sulfate purification, the enzyme had a decreased specific activity of only 50%, with no change in Km value but a tenfold increase in plasma half-life (fig 6). In mice previously immunized to native L-asparaginase, the native enzyme was immediately cleared from the plasma, while the modified enzyme retained a decreased plasma clearance rate. Table 6 shows that it takes a much longer time to observe precipitating antibody when mice are immunized with the modified as opposed to the native enzyme. Through the cooperation of Dr. J. Douros of the National Cancer Institute, Bethesda, Md, we are obtaining enough L-cysteine desulfhydrase to try these modification procedures on this enzyme in order to test the generality of the procedure as well as, hopefully, the therapeutic potential of L-cysteine depletion.

Conclusions

In conclusion, we have reviewed and expanded (table 2) the L-cystine requirements of normal and malignant cells in culture, and have demonstrated that measurements of γ-cystathionase activity may constitute a predic-

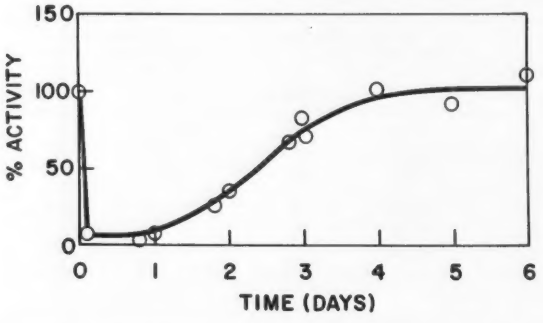

FIGURE 5.—γ-Cystathionase activity from livers of BDF_1 mice injected with a single dose of 14 mg/kg of propargylglycine (16).

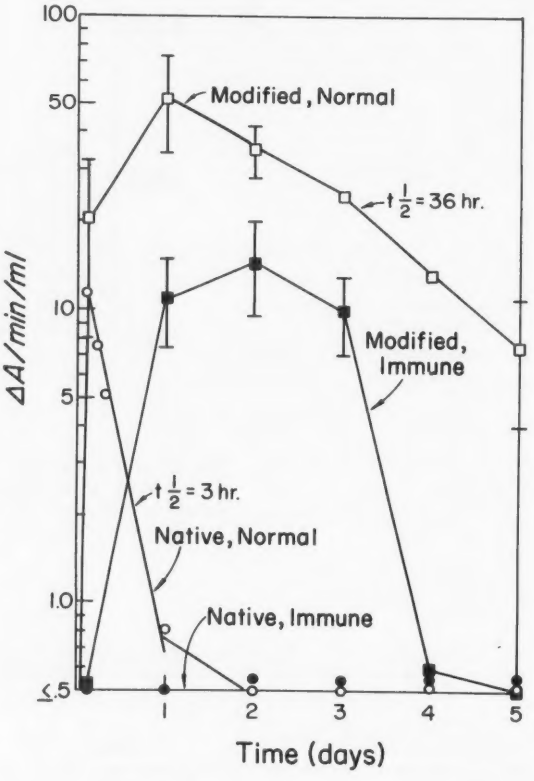

FIGURE 6.—Plasma clearance of native and modified *E. coli* L-asparaginase in normal and immune mice. L-Asparaginase (4 mg/ml) in 0.1 M phosphate buffer, 50% dimethylsulfoxide, pH 8.0, was reacted for 1 hr at 4°C with 30 mg of D,L-alanine-*N*-carboxyanhydride/ml of reaction mixture, desalted with G-25 Sephadex, and ammonium sulfate precipitated between 45% and 60% saturation. 10 IU was injected ip and orbital blood samples were taken at various times and assayed for L-asparaginase activity as described in ref 19. Mice were immunized with 2 injections of DONV-inactivated L-asparaginase as described in the legend to table 6.

TABLE 6.—Immunogenicity of native and modified inactivated L-asparaginase*

Time (wks)	Precipitin antibody	
	Native	Modified
1	–	–
2	+	–
3	+ +	–
4	+ +	+/–

*Each of 20 BDF_1 mice/group were immunized weekly with 20 μg of 5-diazo-4-oxo-L-norvaline (DONV)-inactivated L-asparaginase in complete Freund's adjuvant ip; Ouchterlony precipitin reactions were measured 7 days later. DONV inactivation was performed as described in ref 20.

tive test for tumor sensitivity to L-cyst(e)ine depletion. We have established that L-cysteine desulfhydrase has adequate kinetic properties to inhibit the growth of L1210 in vitro, but is rapidly cleared from animals. We have determined that propargylglycine appears to selectively inhibit L-cysteine biosynthesis in vivo so that possible combination therapy can be envisioned. We have also presented a new chemical-modification procedure for L-asparaginase which greatly increases its plasma half-life and decreases its immunogenicity, which we hope to apply to L-cysteine desulfhydrase.

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γ-Cystathionase in Normal and Leukemic Cells [1]

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SUMMARY

We have now completed further studies into the biochemical basis for L-cysteine auxotrophy in leukemic cells of murine and human origin. We have found that in both species the previously reported decrease in γ-cystathionase activity appears to be due to a reduction in the amount of immunologically normal γ-cystathionase protein. In the human studies, the amount of immunoprecipitable labeled γ-cystathionase protein from cell lines of leukemic origin which display the CYS^- phenotype is decreased. In the murine AKR and Moloney virus-induced leukemias, the reduced enzyme activity correlates with an alteration in the immunoreactivity of γ-cystathionase protein in tumor extracts. The available data are compatible with either of two explanations for the depletion in γ-cystathionase activity which accompanies tumor development: (*a*) the presence of a catalytically inactive, weakly cross-reacting protein product of the γ-cystathionase gene; or (*b*) the progressive reduction in normal γ-cystathionase synthesis, with the residual nonspecific immunoreactivity due to background levels of one or more residual, cross-reactive antigens which have no γ-cystathionase activity and are not products of the γ-cystathionase gene. These alternative possibilities are testable and are presently under investigation. Taken together, the body of evidence strongly suggests that in lymphoid and myeloid cells, malignant transformation may be accompanied by marked reduction of enzymatically active and antigenically normal γ-cystathionase and that this may, at least in part, result in a state of L-cysteine auxotrophy. Our findings with the relatively new cell line, LAZ 221, are of special importance. This line is a bona fide representative of the lymphocytic leukemia cells obtained from a patient with the most prevalent form of acute leukemia. Caution must be exercised in the interpretation of the data obtained with fresh leukemia cells alone, since it is readily apparent that contamination of a mononuclear cell population prepared by Ficoll-Hypaque sedimentation by varying percentages of phagocytes, immature lymphoid cells, or nonhematopoietic cells could affect the results. On the other hand, depressed γ-cystathionase levels and the absence of readily detectable γ-cystathionase protein in LAZ 221 are wholly consistent with the data obtained from fresh leukemic cells, and cannot be the result of contaminating cells. Thus, the findings with this line are likely to closely reflect the situation in the primary leukemia cell in vivo. If these data can be further supported by the examination of individual leukemic blast cells, and if further information regarding the expression of the γ-cystathionase gene in normally developing blast cells shows them to be truly CYS^+ in vivo, the potential exists for a therapeutic approach based on L-cysteine depletion or L-cysteine analog administration.

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The preceding paper reviews our own and other data which indicate that established cell lines from normal and leukemic patients as well as primary leukemic cell cultures from mice differ in their growth requirements for preformed L-cysteine or L-cystine (L-Cys) in culture (1,2). In addition, Ohnuma et al (3) have observed that peripheral blood leukocytes from patients with both acute myelogenous and acute lymphocytic leukemias exhibit an absolute L-Cys requirement to sustain RNA synthesis in short-term cultures. Studies in this laboratory have shown that the L-Cys requirements of both primary tumor cells and well-characterized cell lines may be related to a marked reduction in the amount of enzymatically active γ-cystathionase, the last enzyme in the L-Cys biosynthetic pathway. In the cell lines studied, extracts of those which were L-Cys auxotrophs (CYS^-) contained threefold to 27-fold less enzyme than comparable L-Cys prototrophic cell lines (CYS^+) of similar origin (1). Enzymatic activity was measured in these experiments by a sensitive radioactive assay for the production of [1-^{14}C]-2-ketobutyrate from L-[1-^{14}C]-cystathionine by crude extracts from each cell line.

Based on the findings in established cell lines (1,4) as well as previously published results using an L-Cys analog in patients (5), it would appear that the absolute L-Cys requirement displayed by leukemic cells may render them more sensitive to either depletion of exogenous L-Cys or to cytotoxic L-Cys analogs which they take up more efficiently than normal hematopoietic cells. If this is true, the possibility of using such observations as the basis for a new therapeutic modality for acute leukemia exists. In advance of any such human investigation, we believe that more information regarding the L-Cys requirements of human leukemic cells in vivo as well as the L-Cys requirements of normal tissues is required. To this end, we have recently examined more thoroughly the biochemical basis for the CYS^+ and CYS^- phenotype of human cell lines and murine lymphatic leukemias. These experiments, consisting of sensitive immunoassays, indicate that human CYS^- leukemia cells contain reduced amounts of intact γ-cystathionase protein and that analogous changes occur in primary murine leukemia cells. We have also made preliminary observations in fresh leukemic cells from human bone marrow which suggest that our findings in continuous cell lines and murine lymphatic tumors accurately reflect in vivo changes in human leukemic cells.

γ-Cystathionase Activity in Fresh Human Marrow Mononuclear Cells Obtained From Normal and Leukemic Donors

To date, data obtained from the study of more than ten different cell lines of leukemic origin have demonstrated both an absolute L-Cys growth requirement and depressed γ-cystathionase levels (1). We next turned our attention to the study of fresh leukemic cells to ask whether our findings in cultured cell lines were, at least in part, an accurate reflection of the situation in vivo. Bone marrow cells obtained by aspiration from leukemic or normal persons were purified by Ficoll-Hypaque sedimentation (6). Using this technique, cells which were > 95% pure monocytes (fig 1) were obtained for extraction of enzyme. The crude saline extracts of these cells were then tested for γ-cystathionase activity under standard assay conditions (1).

The results of studies carried out on the marrows of nine normal donors and 17 patients with different forms of acute leukemia are shown in table 1. The mean specific activity of extracts from normal marrow was 0.18 nmol [1-^{14}C]-2-ketobutyrate/mg protein/hour (± 0.13 SD), while the mean specific activity of extracts from the 17 leukemic patients was 0.03 nmol/mg protein/hour (± 0.03 SD) (P = 0.003, Fisher's exact test based on the number of patients with enzyme levels ≤ 0.01). While there is some overlap between the two groups, the data are consistent with a likely explanation for the absolute L-Cys requirements of cultured human and murine leukemic cells previously reported (1) (ie, that leukemic cells may be incapable of synthesizing sufficient L-Cys because of a deficiency in γ-cystathionase). Furthermore, the data suggest that our findings with established cell lines are valid in fresh cells (ie, that the transformed phenotype of certain hematopoietic cells is associated with a reduction in the level of γ-cystathionase relative to levels found in cells of comparable origin which lack the leukemic phenotype).

As a further test of this hypothesis, we have measured γ-cystathionase levels in the bone marrow mononuclear cells of two patients after antileukemic therapy and the achievement of complete or partial remission. Both of these patients were females in their thirties with acute undifferentiated leukemia, and both had γ-cystathionase levels of 0.01 nmol/mg protein/hour at presentation. We found that the specific activity of γ-cystathionase increased threefold to tenfold following remission induction, suggesting that the marrow had become repopulated with cells bearing the normal CYS phenotype. One of the two patients, whose specific γ-cystathionase level rose tenfold (> 0.09 nmol/mg protein/hour), was in complete remission at the time of the second study, while the other, with a threefold rise in γ-cystathionase, had achieved only a partial remission (she relapsed 6 months after the study was completed). These findings represent additional evidence which is consistent with the hypothesis of a link between the leukemic phenotype and reduced γ-cystathionase levels.

Immunochemical Studies of γ-Cystathionase Protein in Human Cell Lines

We next wished to examine cells which were CYS^- and CYS^+ to establish whether the reduced enzyme lev-

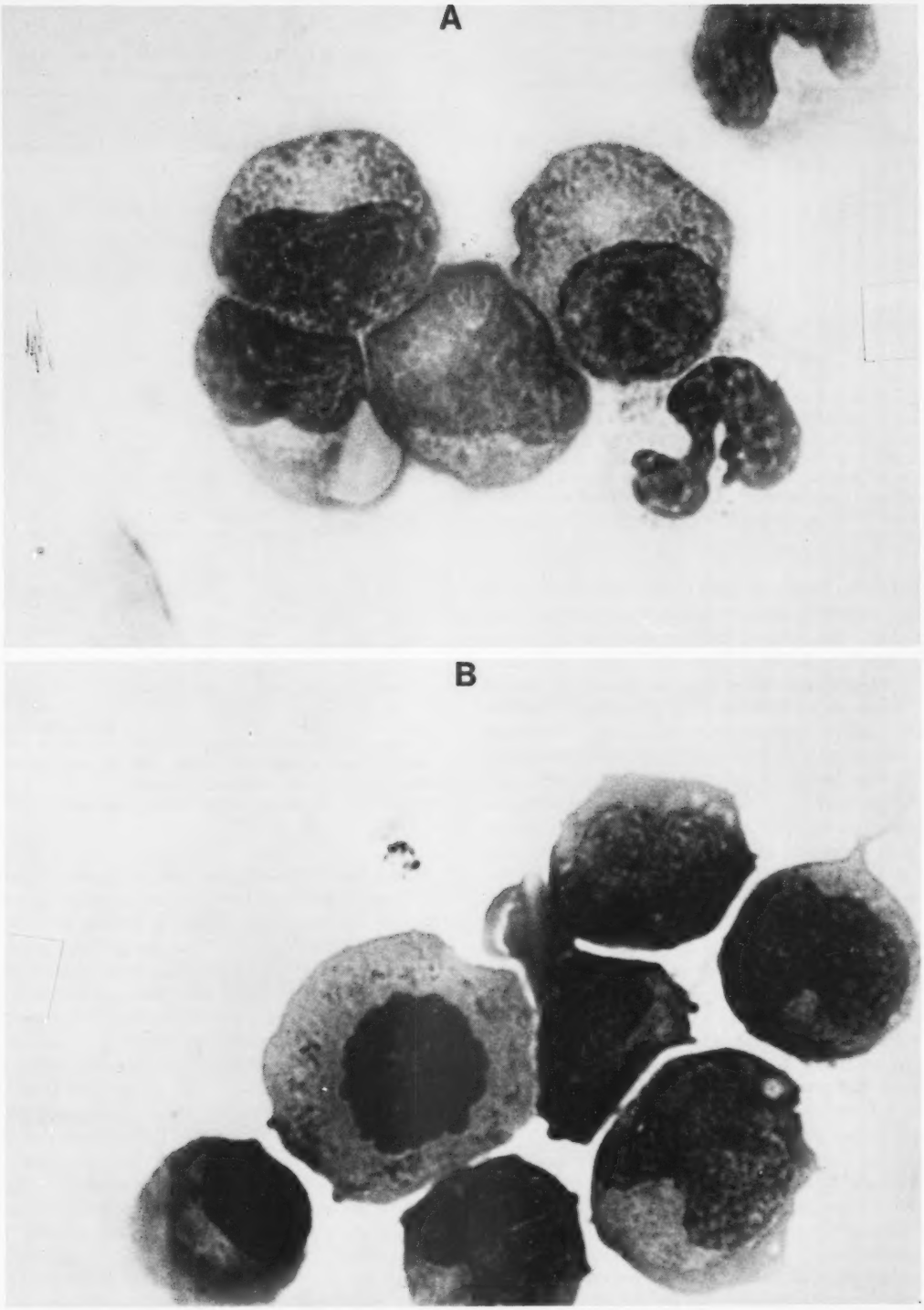

FIGURE 1.—Cytocentrifuge smear of Ficoll-Hypaque purified bone marrow cells from a normal individual (A) and from a patient with acute myelogenous leukemia (B). Wright-Giemsa stain.

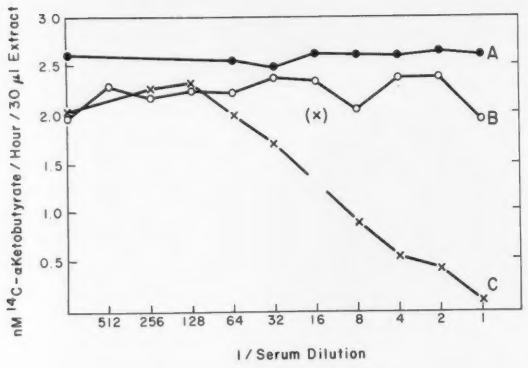

FIGURE 2.—Titration of rabbit anti-γ-cystathionase serum against γ-cystathionase. Increasing dilutions of normal rabbit serum (B) or anti-γ-cystathionase antiserum (A,C) were added to 30 µl of crude rat (A) or human (B,C) liver extracts. After incubation for 1 hr at 37°C and for 18 hrs at 4°C, residual enzymatic activity was determined by the addition of L-[1-^{14}C]-cystathionine and further incubation at 37°C for 1 hr. The [1-^{14}C]-2-ketobutyrate produced was measured by liquid scintillation counting (1).

els found in the former of these cells (1,2) (table 1) were the result of reduced enzyme synthesis or derived from the synthesis of an altered enzyme protein with less enzymatic activity. Based on previously published data, either of these explanations was possible. First, Pascal et al (7) have shown that the very low levels of γ-cystathionase found in fetal and neonatal human liver tissue (8) are correlated with absence of immunochemically detectable γ-cystathionase protein. On the other hand, Frimpter (9) has shown that in certain patients with cystathioninuria, there may be synthesis of an altered apoenzyme with decreased affinity for pyridoxal phosphate. Using a sensitive immunoprecipitation technique, we have now studied a number of the established CYS$^-$ and CYS$^+$ cell lines for the presence of immunologically normal γ-cystathionase.

Human γ-cystathionase was purified from liver by ammonium sulfate precipitation (45%–65% cut), glycerol gradient sedimentation, and isoelectric focusing (1). Highly purified γ-cystathionase obtained in this manner was injected into a New Zealand white rabbit, and an antiserum was produced which formed an immunoprecipitate with purified γ-cystathionase in an Ochterlony double diffusion plate (data not shown).

We tested the ability of this antiserum to neutralize the enzymatic activity found in extracts of human and rat liver. Figure 2 shows the effect of titrating the antiserum against enzyme from each source. The specificity of the antiserum for human γ-cystathionase is shown by the absence of an inhibitory effect of preimmune serum on human γ-cystathionase (curve B), and by the absence of any effect of the antiserum on rat liver enzyme (curve A).

TABLE 1.—Specific activity of γ-cystathionase in extracts of normal and leukemic bone marrow

Patient No.	Diagnosis*	Specific activity† (nmols/mg/hr)
1	Normal	0.29
2	Normal	0.03
3	Normal	0.09
4	Normal	0.06
5	Normal	0.15
6	Normal	0.23
7	Normal	0.38
8	Normal	0.06
9	Normal	0.30
		Mean, 0.18 ± 0.13 (SD)
10	AML	< 0.01
11	AML	0.02
12	AML	0.04
13	AML	0.01
14	AML	0.01
15	AML	< 0.01
16	CML, blastic crisis	0.02
17	CML, blastic crisis	0.08
18	ALL	< 0.01
19	ALL	< 0.01
20	ALL	0.09
21	ALL	< 0.01
22	ALL	< 0.01
23	ALL	0.01
24	ALL	< 0.01
25	ALL	0.08
26	ALL	0.01
		Mean, 0.03 ± 0.03 (SD)

*AML = acute myelogenous leukemia; CML = chronic myelogenous leukemia; ALL = acute lymphocytic leukemia.

†Leukemic vs normal, $P = 0.003$, Fisher's exact test.

Using this antiserum, we then analyzed a number of CYS$^+$ and CYS$^-$ cell lines for the presence of γ-cystathionase by immunoprecipitating labeled protein from crude cell extracts. Figure 3A shows polyacrylamide slab gel bands stained with Coomassie Brilliant Blue. Standard markers, as listed in the figure legend with their respective molecular weights, are shown in slots A and B. Slot C is the material which has been eluted from *Staphylococcus*-A-protein beads. The major visible bands are IgG heavy chain and light chain. Slot D is highly purified human liver γ-cystathionase obtained from an agarose gel slice as described (fig 3A) and acetone coprecipitated with ribonuclease. Human liver γ-cystathionase has been found to have a molecular weight of approximately 190,000 daltons, and probably consists of four subunits of approximately 47,000 daltons (1). In these gels, the γ-cystathionase protein characteristically migrated at a rate slightly faster than the rabbit IgG heavy chain (molecular weight, 49,000 daltons) and at a rate near that of ovalbumin (molecular weight, 43,000 daltons).

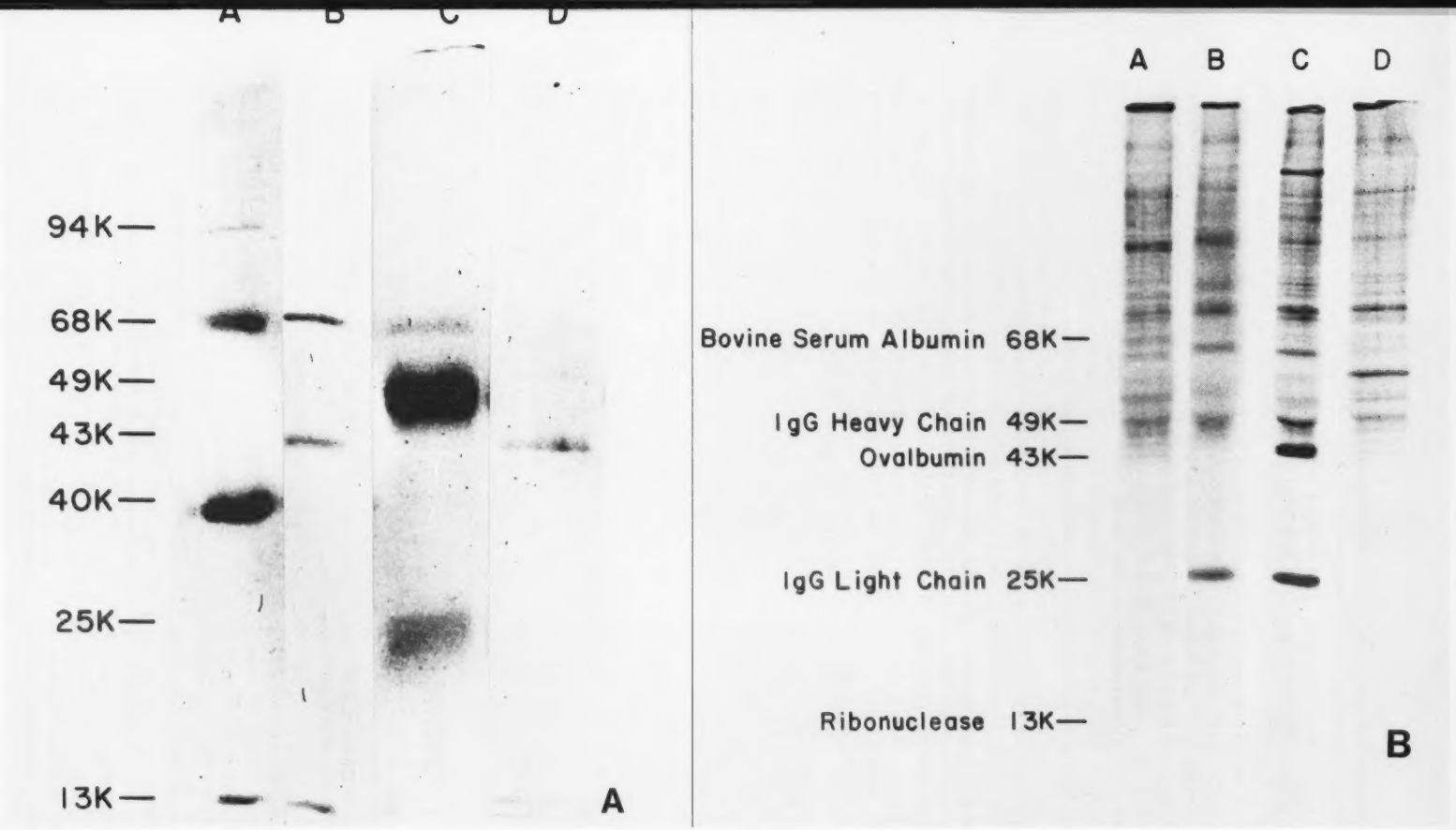

FIGURE 3.—A. Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis of human liver γ-cystathionase and immunoprecipitated proteins from cell lines NC37 and CEM. 2×10^7 cells from each cell line examined were suspended in Eagle's minimum essential medium + 10% dialyzed fetal calf serum and pulsed for 12–18 hrs with 50 microcuries/ml of L-[^{35}S]-methionine (specific activity, 500–600 curies/mmol). The cells were then lysed and labeled proteins were immunoprecipitated by the addition of antiserum and *Staphylococcus*-A-protein linked to Sepharose CL-4B beads as previously described (10,11). The precipitated proteins were then eluted and subjected to SDS polyacrylamide gel electrophoresis under reducing conditions along with homogeneous γ-cystathionase and other standard protein markers. The γ-cystathionase used as a marker in these studies was purified from human liver as previously described (1). Prior to its use as a marker, an active enzyme preparation was further subjected to electrophoresis in a 2% agarose gel and enzyme located using the specific enzymatic stain for deaminating enzymes described by Feldberg and Datta (12). The stained band was then cut from the gel and protein was eluted by electrophoresis into dialysis bags. Proteins stained with 0.2% Coomassie Brilliant Blue in methanol:acetic acid:H_2O (5:1:5). Slots A and B: standard protein markers; phosphorylase—94,000 daltons; bovine serum albumin—68,000 daltons; ovalbumin—43,000 daltons; Aldolase—40,000 daltons; ribonuclease A—13,000 daltons. Slot C: immunoprecipitated proteins from NC37 cell line. Visible bands represent albumin and the heavy (49K) and light (25K) chains of rabbit IgG. Slot D: purified human liver γ-cystathionase (*see* above) and ribonuclease. B. Autoradiograph. 60,000 cpm of labeled, immunoprecipitated proteins from extracts of CEM cells (slots A and B) and NC37 cells (slots C and D) were electrophoresed. Slots A and D are labeled proteins precipitated by normal rabbit serum. Slots B and C are proteins precipitated by rabbit anti-human γ-cystathionase. Gels were exposed to Kodak XR-1 for 72 hrs and developed, and molecular weights were determined by comparison to standard markers (3A).

Figure 3B represents a radioautograph of such a gel. In this case, we precipitated labeled proteins from NC37, a CYS^+ cell line, and CEM, a CYS^- cell line (1). NC37 is a lymphoid cell line consisting of Epstein-Barr virus "transformed" B lymphocytes and is typical of cell lines we have studied which contain easily detectable γ-cystathionase (1). CEM is a well-characterized T-cell leukemia line (CCRF-CEM) which is representative of cell lines which lack γ-cystathionase by enzymatic activity (1). Each slot was loaded with 60,000 cpm of the precipitated, eluted protein. Slot C, which contains the labeled proteins from NC37, has a dark band of activity with a mobility characteristic of the 43,000-dalton molecular weight of the γ-cystathionase subunit. There is little, if any, of this protein detectable in slot B which contains the immunoprecipitated eluted proteins from CEM. Slots A and D represent the respective normal rabbit serum controls for the CEM and NC37 slots. An additional labeled protein with an approximate molecular weight of 25,000 daltons has been detected in both cell lines, as well as in all other cell lines examined. Peptide mapping has shown this protein to be unrelated to γ-cystathionase (data not shown).

Similar experiments have been performed on six additional cell lines. P3J, Ramos, SB, and LAZ 007, each chosen because they contained readily detectable γ-cystathionase in the enzymatic assay, also had an easily detectable 43,000-dalton molecular weight protein in the gels. On the other hand, lines SB2 and LAZ 221 (13), which have markedly diminished enzymatic activity, yielded no detectable 43,000-dalton molecular weight protein. From these studies, we conclude that cell lines which are CYS^- on the basis of their growth requirement for L-Cys and their marked reduction in extractable γ-cystathionase, have reduced concentrations of γ-cystathionase protein.

Immunochemical Studies of γ-Cystathionase in the Mouse

As a model for the human system, we are attempting to examine the relationship between tumorigenesis and γ-cystathionase expression in certain mouse organs. As is the case for the human leukemia cell lines and fresh leukemia cells, thymic lymphomas arising spontaneously in AKR mice or exogenously induced by Moloney type C virus infection in low leukemic mice are characterized by lower levels of γ-cystathionase activity than are found in normal thymic cells (*see* table 3 in the preceding paper by Uren et al in this issue and ref 2).

In order to assess the possibility that the decrease in intracellular γ-cystathionase activity in mouse lymphocytic tumors results from a reduction in the concentration of active enzyme, a measurement of enzyme protein is essential. Therefore, a specific radioimmunoassay (RIA) for mouse γ-cystathionase was developed. This assay has permitted the detection of enzyme protein in the absence of catalytic activity, and has also increased the sensitivity of detection of the enzyme (ie, a major limitation of the best available catalytic assay for γ-cystathionase is the inability to detect the enzyme in a cell-free homogenate of a single mouse thymus). Thus, γ-cystathionase protein concentrations have been measured in a number of mouse tissue homogenates, including normal and spontaneous AKR tumored thymuses. The development of a specific double-antibody RIA and its validation as an accurate measure of mouse γ-cystathionase, as well as of the rat enzyme, is described below.

Mouse liver γ-cystathionase was purified to homogeneity (14) and antisera against it were raised in New Zealand white rabbits. These antisera contained both precipitating and enzyme-neutralizing antibodies. However, when the purified mouse γ-cystathionase was iodinated, there was usually a major loss of immunoreactivity. For this reason, homogeneous rat liver γ-cystathionase (15) was radioiodinated for use as a tracer in the RIA. Immunologic studies with this probe documented that the antisera were capable of precipitating 90% of the labeled protein and that the mouse and rat liver enzymes were immunologically identical (16).

Figure 4 shows the results of competition RIAs for several enzyme preparations using ^{125}I-labeled rat liver γ-cystathionase and a 1:2000 dilution of anti-mouse liver γ-cystathionase antiserum. The competition curves for

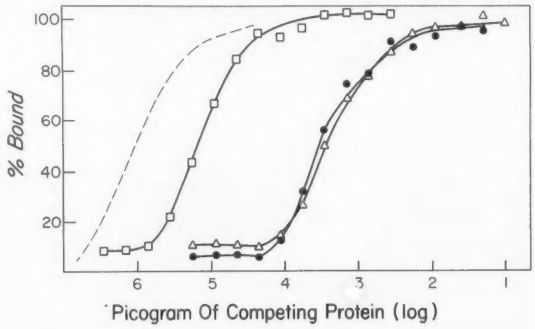

FIGURE 4.—Competition of various γ-cystathionase preparations with ^{125}I-labeled rat liver γ-cystathionase. Samples were diluted in RIA buffer, and sufficient antiserum to precipitate 75% of tracer was added. After incubation for 15 mins at 37°C, 25,000 cpm of tracer was added; following further incubation at 37°C for 1 hr and at 4°C for 18 hrs, antigen-antibody complexes were precipitated by the addition of normal rabbit (NRS) and goat anti-rabbit IgG. Data are expressed as:

$$\frac{\text{cpm precipitated by immune serum} - \text{cpm precipitated by NRS}}{\text{total cpm precipitated by trichloroacetic acid}} \times 100.$$

● = purified mouse liver γ-cystathionase; △ = purified rat liver γ-cystathionase; □ = mouse liver γ-cystathionase purified through ethanol precipitation step (16); - - - - = supernatant fluid from crude mouse liver homogenate.

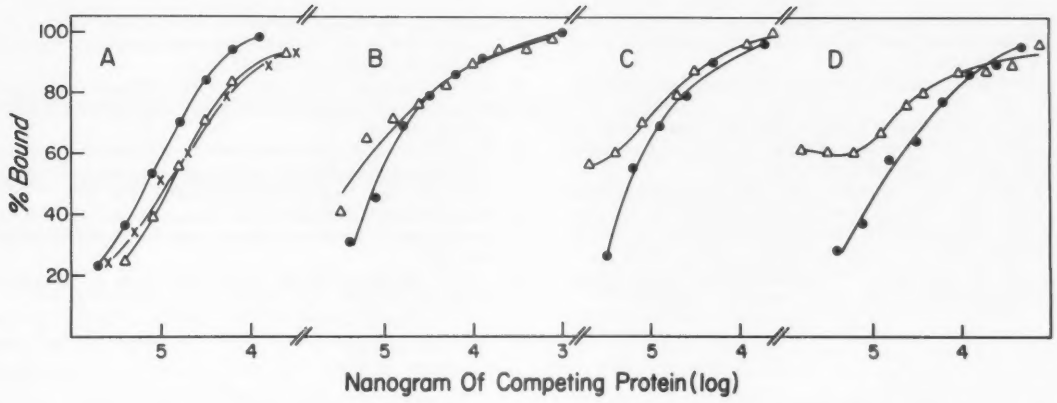

FIGURE 5.—Double-antibody competition RIAs for γ-cystathionase in the supernatant fluids from homogenates of AKR mouse thymuses and thymic tumors. A. 2-mo-old AKR thymus (●); 4-mo-old AKR thymus (△); 5-mo-old AKR thymus (×). B. 7-mo-old AKR thymus (●); 7-mo-old AKR spontaneous thymoma (△). C. 9-mo-old AKR thymus (●); 9-mo-old AKR spontaneous thymoma (△). D. 10-mo-old AKR thymus (●); 10-mo-old AKR spontaneous thymoma (△).

purified rat and mouse liver γ-cystathionase are virtually superimposable, indicating that the two enzymes contain common antigenic determinants, thus validating the use of the labeled rat enzyme for the measurement of mouse γ-cystathionase. With this system, 1 ng of either enzyme can be detected readily. Also shown in figure 4 are competition curves for crude mouse liver homogenate and for mouse liver γ-cystathionase partially purified through an ethanol-precipitation step (14). The similarity of the slopes for these displacement curves to those for the two purified enzymes is apparent.

Immunoreactivity of γ-Cystathionase in Thymuses of Normal and Leukemic Mice

AKR mice are characterized by an incidence of lymphoma of approximately 90% by 10 months of age. These mice show gross pathologic evidence of neoplastic transformation, occurring first in the thymus (17), and beginning at 7–8 months of age. Most of these animals subsequently die of the disease at 9–10 months of age (compared to a mean lifespan of 22 months for C57BL/6 mice, a low virus and low leukemia incidence strain).

We wished to determine whether in this model system, decreased levels of γ-cystathionase activity in the thymic tumors (2) correlated with lower levels of enzyme protein, or were due to a structurally altered enzyme, or both.

Figure 5 shows the results of competition RIAs for γ-cystathionase in thymuses from healthy and leukemic AKR mice; thymuses from the latter mice were grossly enlarged. Extracts of 7-, 9-, and 10-month-old spontaneous AKR mouse thymomas were tested in the RIA along with extracts of age-matched thymuses which were grossly tumor-free. The series of curves show that with increasing age, the immunoreactive γ-cystathionase from tumored thymuses becomes increasingly dissimilar compared to that from normal thymuses. Additional studies, in which equal amounts of proteins from tumored and normal thymuses were assayed after incubation together, made it unlikely that the altered antigenic structure was due to proteolysis in the tumor (data not shown). Thus, it appears that with progressive replacement of normal thymus cells by leukemic cells, there is a simultaneous loss of antigenically normal γ-cystathionase. These results suggest that the reduction in enzymatic activity which accompanies the development of thymic tumors in AKR mice (2) may be explained by a decrease in the concentration of structurally normal γ-cystathionase protein.

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L-Threonine Deaminase as a Possible Antitumor Agent[1]

Daniel Wellner[2,*] and Robert S. Greenfield[2,3]

SUMMARY

In vitro studies with L-threonine deaminase purified from sheep liver demonstrated that the enzyme was cytotoxic toward two lines of murine leukemia cells but not toward L cells or normal human fibroblasts. When L-threonine deaminase was injected into mice, it was possible to lower the plasma L-threonine concentration by 90%. The enzyme had a half-life of 1.5 hours in the circulation.

[Cancer Treat Rep 63:1089–1094, 1979]

It has been 25 years since Kidd (1) discovered the antineoplastic properties of guinea pig serum. This discovery opened an entirely new field of investigation: namely, the use of enzymes as drugs for the treatment of cancer. The active component of guinea pig serum was shown to be L-asparaginase (2), an enzyme which catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. L-Asparaginases from bacterial sources were also shown to possess antitumor activity (3). Although L-asparaginase at first appeared very promising in the treatment of acute lymphoblastic leukemia, serious problems have been encountered in its use. The principal problem is the appearance of resistant cancer cells. The resistant cells have been shown to possess large amounts of L-asparagine synthetase, an enzyme which is also present in most normal tissues (4,5). The presence or inducibility of L-asparagine synthetase in these cells appears to account for their resistance to L-asparaginase treatment.

We thought that it would be worthwhile to explore the potential of other amino acid-metabolizing enzymes for their ability to deplete specific amino acids from the plasma and for their antitumor activity.

The first enzyme tested for antitumor activity in our laboratory was the L-amino acid oxidase of snake venom (6). This enzyme catalyzes the oxidation of a number of amino acids to the corresponding α-keto acid and ammonia. When this enzyme was highly purified, it could be injected into mice in relatively large doses with little or no toxic effects. (Its co-enzyme, FAD, is tightly bound and need not be added for optimal activity.) The enzyme had a half-life of several hours in the circulation, and decreased the level of a number of amino acids in plasma to very low levels. Significant decreases in the plasma concentration of L-methionine, L-leucine, L-tyrosine, L-phenylalanine, and L-glutamic acid were observed (6). When we did experiments involving injections of this enzyme into tumor-bearing animals, we found no significant effects on tumor growth or on the lifespan of the animals. We attributed this lack of effect to the ability of cells to reconvert the α-keto acid to the original amino acid by means of transamination reactions.

We then decided to study L-threonine deaminase for two principal reasons: (*a*) the products of the enzymatic reaction cannot be utilized by the cell for L-threonine synthesis, and (*b*) L-threonine is a dietary essential amino acid which cannot be made by mammalian cells. Since mammalian cells presumably do not possess the genetic coding information for the enzymes needed for L-threonine synthesis, there is no possibility of these enzymes appearing and the cells thereby becoming resistant to treatment with L-threonine deaminase.

L-Threonine deaminase catalyzes the conversion of L-threonine to α-ketobutyrate and ammonia. The enzyme from sheep liver was chosen for the initial studies because (*a*) it is very stable (it can be heated to 72°C with no loss of activity); (*b*) its co-factor, pyridoxal-5′-phosphate, is quite tightly bound; (*c*) in contrast to the bacterial enzymes, it is not allosteric; (*d*) it has a relatively low molecular weight of about 53,000; and (*e*) it is very active under the physiologic conditions of blood plasma. We have obtained the enzyme in a highly purified state and

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studied some of its properties (7). The purification consists of two heat steps, two ammonium-sulfate fractionations, and chromatography on DEAE- and CM-cellulose columns. There are several peaks of activity which elute from the final CM-cellulose chromatographic column. These represent some of the seven isozymes we have observed by isoelectric focusing of the enzyme. The experiments discussed here were done with a mixture of isozymes.

We tested the effects of L-threonine deaminase on four different cell lines in tissue culture (8). Two of these were radiation-induced murine leukemias and the other two were mouse L cells and normal human fibroblasts. Figure 1 shows the effects of L-threonine deaminase on the growth and viability of EARAD-1 leukemia cells, an L-asparaginase-sensitive line. In the presence of the enzyme (0.26 unit/ml), the cells did not grow and were killed within 24 hours, as determined by trypan blue uptake. Inactivated enzyme was added in the control flasks.

A similar experiment was done with RADA-1 cells, an L-asparaginase-resistant strain. The results are shown in figure 2. Curves *e* and *f* had 0.26 and 1.3 units/ml of L-threonine deaminase, respectively. In the experiment represented by curve *d*, no enzyme was added, but the cells were in an L-threonine-free medium. In all of these experiments, the cells were killed within 48 hours. In the presence of the enzyme, the cells lost viability somewhat faster than in the L-threonine-free medium.

Figure 3 shows the effect of varying the L-threonine deaminase concentration on the rate of viability loss and on the L-threonine concentration of the medium. At 0.065 unit/ml of L-threonine deaminase (open circles), the cells did not begin to lose viability until 24 hours after enzyme addition; at lower enzyme concentrations, they remained viable.

Simultaneous determinations of L-threonine levels in the medium indicate that the cells do not survive at L-threonine concentrations < 20 μM.

As shown in figure 4, mouse L cells also stopped growing in the presence of L-threonine deaminase. However, there was little or no loss of viability in 96 hours. When the medium was replaced with fresh medium containing no enzyme, the cells started to divide again.

With normal human fibroblasts there was no growth but also no loss of viability for at least 5 days in the presence of L-threonine deaminase (8). In this case, the cells did not resume growth on replacing the medium, but they did grow following trypsinization and replating in fresh, L-threonine-containing medium.

The experiments shown in figure 5 were carried out in an attempt to demonstrate the possibility of significantly lowering the plasma L-threonine concentration by injections of purified L-threonine deaminase. It can be seen that a single injection of 10 units of L-threonine deaminase in a mouse was sufficient to lower the plasma

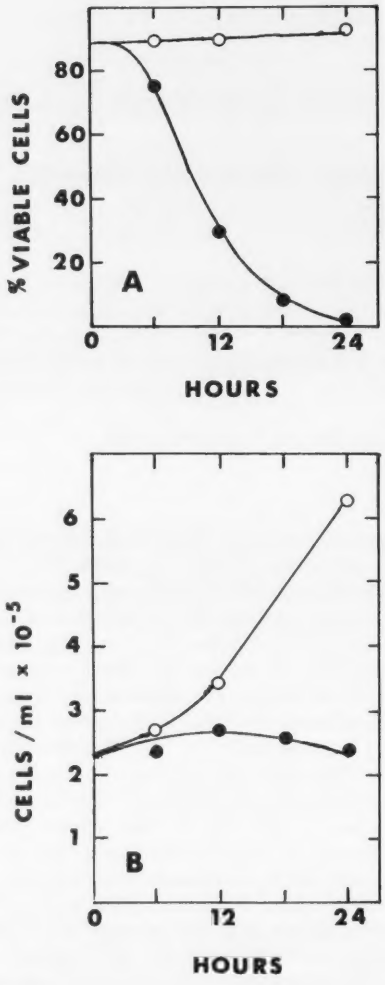

FIGURE 1.—Effects of L-threonine deaminase on growth and viability of EARAD-1 murine leukemia cells in vitro. ● = 0.26 unit/ml of L-threonine deaminase; ○ = same amount of inactivated enzyme (*from* ref 8).

L-threonine level by about 90% in 2 hours before it gradually returned to normal in 24 hours. A second injection of enzyme prolonged the L-threonine depletion. As shown in figure 6, L-threonine deaminase had a half-life in the circulation of about 1.5 hours.

The studies described here suggest that L-threonine deaminase is a potentially useful anticancer agent. Additional studies, both in vitro and in vivo, are needed to gain a better understanding of the effects of this enzyme on tumors and on normal tissues and cells. We do not understand what properties of cells are important in determining whether they will remain viable or not under

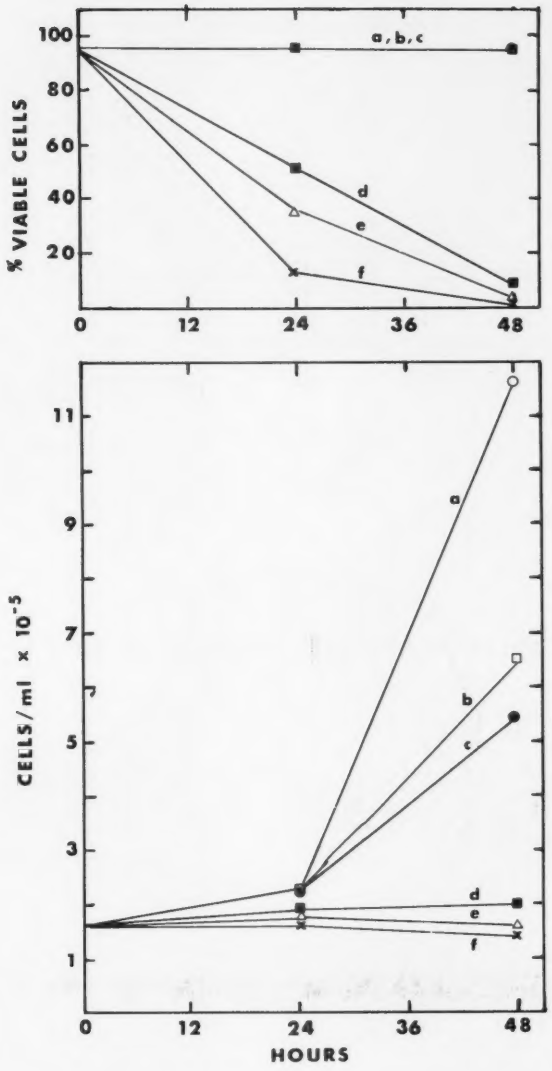

FIGURE 2.—Effects of L-threonine deaminase on growth and viability of RADA-1 murine leukemia cells in vitro. *a* and *b* = L-threonine deaminase (0.26 and 1.3 units/ml, respectively) inactivated with sodium borohydride; *c* = no additions; *d* = no additions, L-threonine-free medium; *e* and *f* = L-threonine deaminase (0.26 and 1.3 units/ml, respectively) (*from* ref 8).

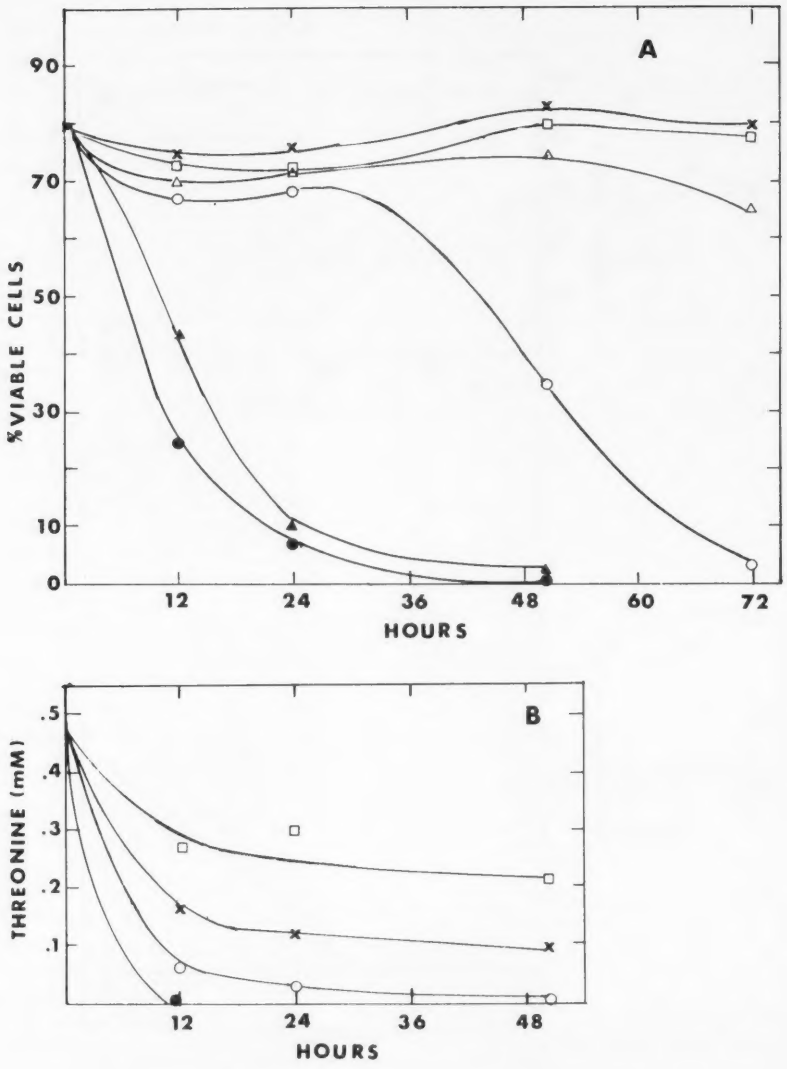

FIGURE 5.—Correlation between cytotoxic effect of L-threonine deaminase on EARAD-1 cells and L-threonine levels in the medium. Additions: ● = L-threonine deaminase, 0.26 unit/ml; ▲ = 0.13 unit/ml; ○ = 0.065 unit/ml; × = 0.032 unit/ml; □ = 0.013 unit/ml; △ = inactivated enzyme (*from* ref 8).

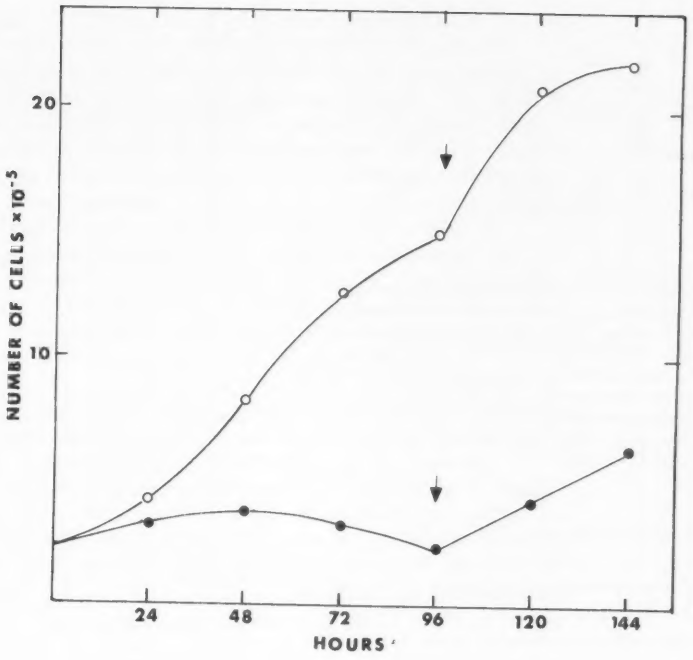

FIGURE 4.—Growth inhibition of mouse L cells by L-threonine deaminase. Additions: ● = L-threonine deaminase (0.26 unit/ml); ○ = inactivated enzyme. Arrows indicate time when cells were washed twice and placed in fresh media with no enzyme (*from* ref 8).

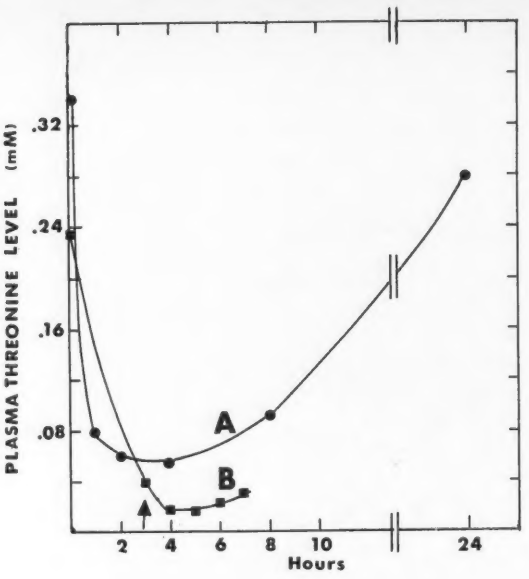

FIGURE 5.—Plasma L-threonine concentrations following ip injections of L-threonine deaminase in mice. A = 1 ml of solution containing 10 units of purified L-threonine deaminase (specific activity: 1 unit/mg) was injected at Time 0 into a C57BL female mouse. Blood samples were obtained by periorbital bleeding with heparinized pipettes at indicated times. Plasma L-threonine concentrations were determined after deproteinization with sulfosalicylic acid on a Durrum Model 500 amino acid analyzer. B = same as A except that a 2nd injection of enzyme (10 units) was made after 3 hrs (arrow).

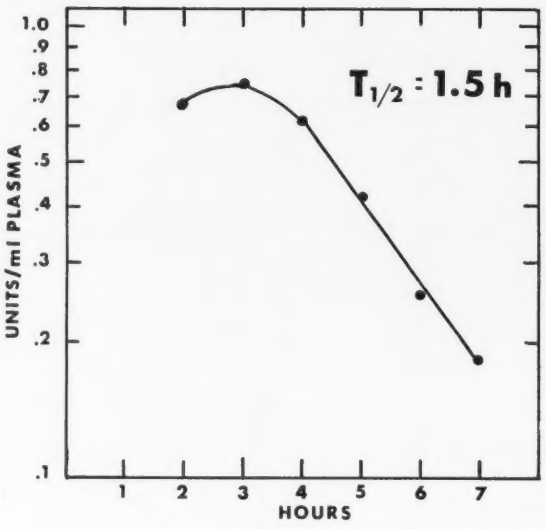

FIGURE 6.—Determination of L-threonine deaminase half-life in mouse plasma in vivo. Enzyme was injected as in fig 5. Blood samples were obtained at indicated times and plasma was assayed for L-threonine deaminase activity.

conditions of amino acid deprivation. The studies of Pardee and James (9,10), Holley and Kiernan (11), Tobey and Ley (12), and others seem to be relevant to this problem. These investigators find that "normal" cells, when deprived of an essential nutrient, become arrested in the G_1 phase of the cell cycle and enter a quiescent state in which they remain viable for prolonged periods of time. Many malignant or transformed cells, on the other hand, continue to proliferate and remain metabolically active, resulting in their death under such adverse conditions.

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Analogs of L-Aspartic Acid in Chemotherapy for Cancer

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SUMMARY

The interaction of analogs of L-aspartic acid with adenylosuccinic acid synthetase, L-asparagine synthetase, and L-aspartic acid transcarbamylase is discussed. Each of these enzymes is of critical importance to the economy of certain types of tumor cells. L-Alanosine, a new antitumor antibiotic, is shown to be accepted as a substrate by the enzymes of de novo purine biosynthesis which ordinarily use L-aspartic acid as a substrate; as a consequence of this interaction, an anabolite is thought to be produced which impairs the formation of adenine nucleotides by inhibiting adenylosuccinate synthetase, leading to an interruption in DNA synthesis. Homoserine-β-adenylate, guanidinosuccinic acid, and PA_2LA [3-(phosphonacetylamido)-L-alanine] are shown to be inhibitors of L-asparagine synthetase from murine lymphoblasts; each of these analogs of L-aspartic acid exhibits novel structural properties which can be used by synthetic chemists in the design of molecules with an even greater ability to block the biosynthesis of L-asparagine. Certain aspects of the mechanism of action of PALA (*N*-phosphonacetyl-L-aspartic acid) were examined. This agent, which is a potent inhibitor of mammalian L-aspartic acid transcarbamylase, is capable of stimulating the homologous enzyme from *Escherichia coli* under certain circumstances. In vivo the duration of inhibition produced by this agent is shown to be unusually protracted; for example, L-aspartic acid transcarbamylase in mouse liver remains at 30% of treatment levels for ≥ 20 days after a single therapeutic dose of PALA. This long-lasting effect reflects either sluggish synthesis of new enzyme molecules in this organ or shuttling of the inhibitor from old to new molecules. It is suggested that new and still more potent analogs of L-aspartic acid be sought, and that they be screened, inter alia, against these target enzymes.

[Cancer Treat Rep 63:1095–1108, 1979]

Analogs of L-aspartic acid merit development as antitumor agents because they interact profitably with three enzymes important to tumors: (*a*) adenylosuccinic acid synthetase (EC 6.3.4.4), (*b*) L-asparagine synthetase (EC 6.3.1.1), and (*c*) L-aspartic acid transcarbamylase (EC 2.1.3.2). Before each of these enzymologic areas is considered, a word of background is in order.

Generally, the most potent amino acid antagonists with antitumor activity are those which disturb the synthesis of nucleic acids. Only three amino acids are involved in the biosynthesis of the purine and pyrimidine rings. They are glycine, L-glutamine, and L-aspartic acid. Antagonists of glycine are rare and not especially noted for their antitumor or antimetabolic activity.[3] Oncolytic antagonists of L-glutamine, on the other hand, are plentiful and potent; the latest of these antagonists slated for clinical tests, chloroisoxazole (NSC-163501), is described in detail elsewhere (1,2) and in these proceedings. In comparison to this large family of drugs, the antagonists of L-aspartic acid are neglected. L-Aspartic acid is, of course, a non-

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[3]Halacidin (*N*-formyl-*N*-hydroxyglycine), an antagonist of glycine, does exhibit marginal antineoplastic activity.

essential amino acid, but it does play an indispensable role in the construction of both the purine and pyrimidine rings. In pyrimidine biosynthesis its whole carbon skeleton is used. (More will be said of this later.) In purine biosynthesis it gives up its amino nitrogen at two critical points, as indicated in figure 1:

1. In the formation of 5-aminoimidazole-4-*N*-succinocarboxamide ribonucleotide, otherwise known as SAICAR. The operative enzyme here is known by the trivial name, SAICAR synthetase (EC 6.3.2.6).
2. In the formation of succinyl IMP, otherwise known as adenylosuccinic acid. The operative enzyme here is adenylosuccinate synthetase.

In both cases, the carbon skeleton of L-aspartic acid is transitorily bonded to the nascent purine ring through its α-amino nitrogen. These carbon atoms are then cleaved by a bifunctional enzyme, adenylosuccinate lyase (EC 4.3.2.2), to yield fumaric acid and a new purine enriched in a nitrogen functionality.

Inhibition of Adenylosuccinate Synthetase by L-Alanosine

L-Alanosine is a new amino acid which is believed to interact at both of these steps in its capacity as an antagonist of L-aspartic acid. Elaborated by *Streptomyces alanosinicus*, it shows good activity against several transplantable murine tumors, including P388 and L5178Y leukemias (3,4). Viewed side by side with L-aspartic acid, it is clear that L-alanosine and L-aspartic acid have similar shapes; the *N*-nitroso function is nearly superimposable on the carbonyl group (fig 2). Moreover, substantial enzymatic evidence suggests that L-alanosine is a functional analog of L-aspartic acid in certain systems. For example, L-aspartate aminotransferase, adenylosuccinate synthetase, and SAICAR synthetase all accept it as a substrate (table 1). As a consequence of this last interaction, L-alanosine can be anabolized to yield the structure shown in figure 3. This conjugation was first reported in 1977 by Hurlbert et al (5). At about the same time, studies in our laboratory demonstrated that L-alanosine was a competitive inhibitor, against L-aspartic acid, of the SAICAR synthetase reaction, with a K_i of 1.6×10^{-3} M (fig 4). The two sets of data are, therefore, complementary. These findings are enzymologically interesting but not surprising on structural grounds. What proved to be surprising, however, was the kinetics of inhibition by L-alanosine of the second enzyme utilizing L-aspartic acid

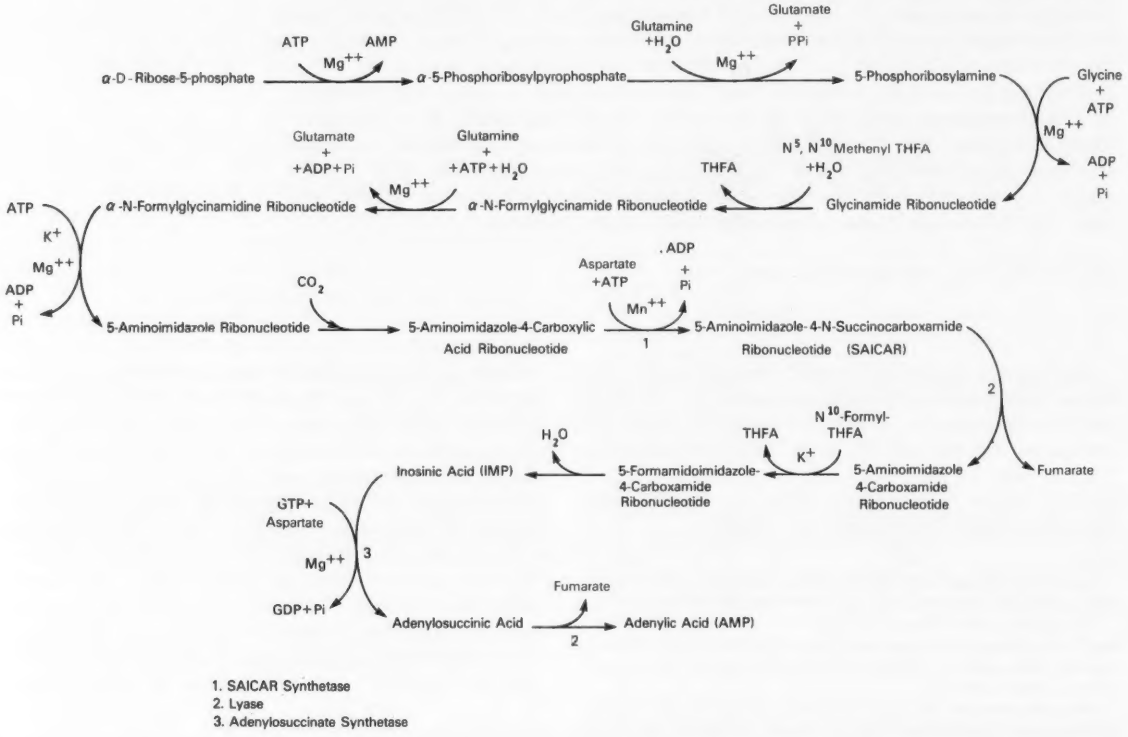

FIGURE 1.—Summary scheme of AMP biosynthetic pathway.

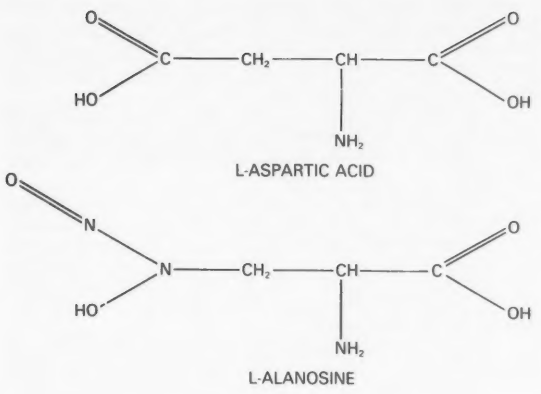

FIGURE 2.—Chemical structures of L-aspartic acid and L-alanosine.

in the course of purine biosynthesis, adenylosuccinic acid synthetase. As previously mentioned, the mechanisms of action of this enzyme and of SAICAR synthetase are quite comparable. A priori, therefore, we anticipated competitive kinetics with L-aspartic acid as a variable substrate in both cases. Instead, L-alanosine was found to produce noncompetitive inhibition of adenylosuccinate synthetase (fig 5). L-Alanosine is known to stop the biosynthesis of AMP in vivo and, on several lines of evidence, does so at the very step in question, ie, by inhibiting adenylosuccinic acid synthetase (6). Thus, the pools of AMP, but not GMP, fall after exposure of tumor cells to L-alanosine in vitro (7). This selective fall means that the drug is not inhibiting the assembly of the purine ring, inasmuch as AMP and GMP share this ring; rather, it must be influencing final modifications made on the ring and restricted to the adenine nucleotide pathway. It follows, then, that the competitive inhibition by L-alanosine of SAICAR synthetase, the early enzyme catalyzing the formation of both nucleotides, is irrelevant to its pharmacologic action. The critical point of attack had to be at the level of adenylosuccinic acid synthetase or lyase. Since L-alanosine fails to inhibit adenylosuccinic acid lyase either in vivo or in vitro, this enzyme could be discounted as a point of attack.[4] A problem still remained. The K_i of L-alanosine versus adenylosuccinic acid synthetase (5×10^{-3} M) seemed far too high to explain the pharmacologic potency of L-alanosine; about 5×10^{-7} M is an ID50 to susceptible cells in culture. Hurlbert et al (5) found the following explanation for this disparity: the anabolite resulting from the condensation of AICOR with L-alanosine catalyzed by the first L-aspartyl enzyme of purine biosynthesis, SAICAR synthetase, functions, in fact, as an extremely potent inhibitor of the second L-aspartyl enzyme of purine biosynthesis, adenylosuccinic acid synthetase. In other words, it is "alanosyl AICOR" that inhibits adenylosuccinic acid synthetase at a pharmacologically meaningful concentration. In preliminary experiments, we have confirmed Hurlbert's finding, isolated a modicum of this inhibitor, and estimated its K_i to be $< 1 \times 10^{-6}$ M.

Another interesting point is that, like SAICAR and adenylosuccinic acid, the presumptive alanosyl adduct, "alanosyl AICOR," is thought to be decomposed by adenylosuccinic acid lyase (fig 6). L-Alanosine has just entered clinical trials; for this reason, it has become increasingly important to measure the specific activities of SAICAR synthetase, adenylosuccinic acid synthetase, and adenylosuccinic acid lyase in tumor biopsies. Briefly, tumors rich in SAICAR synthetase but deficient in the lyase, or in adenylosuccinic acid synthetase, ought to be ideal candidates for a response.

[4]We have not yet determined whether the adduct of L-alanosine with IMP, whose production is catalyzed by adenylosuccinic acid synthetase, can behave as an inhibitor of adenylosuccinic acid lyase. Some support for this idea comes from the work of Brand and Lowenstein with adducts of IMP and β-phosphono-L-alanine (8); these molecules, which are structurally quite close to "alanosyl-IMP," are exceedingly potent inhibitors of the lyase, with K_is of 1×10^{-9} M.

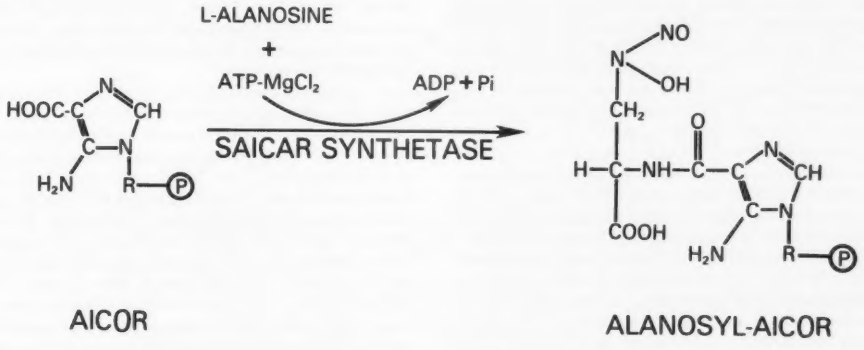

FIGURE 3.—Postulated interaction of L-alanosine with SAICAR synthetase.

TABLE 1.—Interaction of L-alanosine with the enzymes of L-aspartate metabolism

Enzyme	L-Alanosine as —	
	Substrate	Inhibitor (ID50 in mM)
1. Adenylosuccinate synthetase	+	7
2. Argininosuccinate synthetase	–	30
3. L-Aspartate transcarbamylase	+	40
4. SAICAR synthetase	+	1.6
5. L-Aspartyl-tRNA synthetase	–	5
6. L-Asparagine synthetase	–	—
7. L-Aspartate-*N*-acetylase	–	—
8. L-Aspartate aminotransferase	+	0.5
9. L-Aspartase	–	—
10. L-Aspartate-β-decarboxylase	–	—
11. L-Aspartyl kinase (*E. coli*)	–	—
12. L-Aspartate transport enzyme	+	5.5

1. *Adenylosuccinate synthetase:* activity was measured in a reaction mixture of 20 µl containing 5 µl of L-[4-^{14}C]-aspartic acid (6.37 nmols, 125 nanocuries); 5 µl of GTP-$MgCl_2$ (150 nmols each); 5 µl of IMP (150 nmols) or Tris-HCl buffer, pH 8.0 (250 nmols); 5 µl of water or L-alanosine; and 5 µl of enzyme extract.

2. *Argininosuccinate synthetase:* in a volume of 20 µl were admixed: 250 nanocuries (12.74 nmols) of L-[4-^{14}C]-aspartic acid; 50 nmols of citrulline; 50 nmols of ATP-$MgCl_2$ in 0.05 M Tris-HCl, pH 8.0, or buffer alone; 5 µl of water or L-alanosine; and, to initiate the reaction, 5 µl of mouse kidney extract.

3. *L-Aspartate transcarbamylase:* in a total volume of 20 µl were admixed: 250 nanocuries (12.74 nmols) of L-[4-^{14}C]-aspartic acid; 5 µl of water or L-alanosine; 100 nmols of carbamyl phosphate; and, to initiate the reaction, 5 µl of mouse spleen extract.

4. *SAICAR synthetase:* in a total volume of 30 µl were admixed: 250 nanocuries (12.74 nmols) of L-[4-^{14}C]-aspartic acid; 86 nmols of SAICAR; 250 nmols of ATP-$MgCl_2$; 500 nmols of sodium phosphate buffer, pH 7.3; 5 µl of water or L-alanosine; and, to initiate the reaction, 5 µl of partially purified chicken liver enzyme.

5. *L-Aspartyl-tRNA synthetase:* aminoacetylation was carried out in a reaction volume of 100 µl containing 50 mM Tris-HCl, pH 7.2; 2 mM $MgCl_2$; 15 mM KCl; 0.25 mM EDTA; 5 mM ATP; 50 µg of tRNA; 0.25 microcurie of L-[U-^{14}C]-amino acid; and 50 µl of the enzyme preparation. After incubation at 37°C for 30 mins, 10% TCA was added to arrest the reaction. The precipitated macromolecules were collected on glass fiber filters (Whatman 34-AH), washed 3 times with cold 5% TCA and once with 95% ethanol, and dried; radioactivity was measured by scintillation spectrometry.

6. *L-Asparagine synthetase:* freshly excised L5178Y tumor nodules resistant to L-asparaginase (L5178Y/AR) were homogenized in 3 volumes (wt/vol) of 0.1 M Tris, pH 7.6, containing 0.5 mM EDTA and 1 mM dithiothreitol. The homogenate was centrifuged at 12,000 × *g* for 3 mins and the clear supernatant was used as the source of L-asparagine synthetase. The reaction mixtures, in a net volume of 45 µl, consist of 3 nmols (0.07 microcurie) of L-[4-^{14}C]-aspartic acid; 1 µmol of L-glutamine or 2.5 µmols of NH_4Cl; 1.5 µmols of ATP and $MgCl_2$; 2.5 µmols of Tris-HCl buffer, pH 8.4; and 5 µl of L-alanosine or other inhibitors at the indicated concentration, along with 5 µl of crude enzyme from L5178Y/AR tumor. The reactants were mixed by centrifugation and incubated at 37°C for 30 mins, after which the reaction was terminated by heating at 95°C for 5 mins. In order to remove unreacted L-[4-^{14}C]-aspartic acid, an enzymatic reagent consisting of 50 µl of 0.66 M sodium acetate buffer, pH 5.0, containing 6 mM α-ketoglutaric acid, 8.5 mM $ZnSO_4$, and 1 unit of L-glutamate oxaloacetate transaminase, was added to the reactants and the open reaction vessels were incubated at 37°C for 4 hrs. At this point, 2 IU of *E. coli* L-asparaginase was added to the apex of each vessel, a droplet of 40% KOH (wt/vol) was deposited on the underside of the lid, and the vessels, after capping, were incubated at 37°C for 3 hrs. The $[^{14}C]O_2$ generated was trapped by the KOH droplet. The lids were removed and immersed in a scintillation mixture of ethanol-toluene (1:2 vol/vol) containing Liquifluor and the radioactivity was counted in a Beckman LS-230 liquid scintillation spectrometer at 87% efficiency.

7. *L-Aspartate-N-acetylase:* in a total volume of 25 µl were admixed: 250 nanocuries of L-[4-^{14}C]-aspartic acid; 50 nmols of acetyl coenzyme A; 5 µl of water or L-alanosine; and, to initiate the reaction, 5 µl of brain homogenate.

In the case of enzymes 1–7, the reactants were mixed by centrifugation and incubated at 37°C for 30 mins, after which the reaction was terminated by heating at 95°C for 2 mins. Excess L-[4-^{14}C]-aspartic acid was dissipated by adding 50 µl of decarboxylation reagent (*see* 6 above). The open vessels were incubated at 37°C for 3 hrs, and then, in the case of enzymes 1–5 and 7, immersed in a liquid scintillant and counted in a Beckman LS-230 spectrometer.

8. *L-Aspartate aminotransferase:* in a final volume of 25 µl were admixed: 5 µl of L-[U-^{14}C]-glutamic acid (0.25 microcurie, 215 nmols); 5 µl of 0.05 M Tris-HCl buffer, pH 7.6; 5 µl of 0.03 M oxaloacetic acid; 5 µl of L-alanosine (0.1–1.0 mM) or water; and 5 µl of L-glutamate oxaloacetate transaminase from pig heart (0.02 mg/ml). The reactants were mixed by centrifugation, incubated at 37°C for 30 mins, and then heated at 95°C for 2 mins. The α-ketoglutaric acid formed was measured by decomposing it with 1% H_2O_2 in 1.0 N HCl and collecting the $[^{14}C]O_2$ formed for 1 hr in drops of 40% KOH.

9. *L-Aspartase:* crude L-aspartase from *Bacillus cadaveris* or *E. coli* was dissolved in 0.05 M Tris-HCl, pH 8.4, to a final concentration of 1 mg/ml. To determine if L-alanosine was capable of inhibiting L-aspartase, 5 µl of the enzyme was incubated with 5 µl (0.25 microcurie) of L-[U-^{14}C]-aspartic acid and 5 µl of 0.05 M Tris-HCl, pH 8.4, or L-alanosine at final molarities ranging from 1 to 20 mM. Fumaric acid was measured by paper electrophoresis. To determine if L-alanosine could serve as a substrate for L-aspartase, 5 µl of enzyme was incubated at 37°C with 5 µl (0.24 microcurie) of DL-[1-^{14}C]-alanosine for 30–300 minutes. Aliquots of the incubation mixture were then either electrophoresed or subjected to high-resolution column chromatography on Hamilton HA-X4 resin.

10. *L-Aspartate-β-decarboxylase:* to measure the ability of L-alanosine to inhibit the β-decarboxylation of L-aspartic acid, 5 µl (0.25 microcurie) of L-[U-^{14}C]-aspartic acid was incubated with 5 µl (1 IU) of L-aspartate-β-decarboxylase from *Alcaligenes faecalis* in 0.66 M sodium acetate, pH 4.8, in the presence or absence of 5 µl of 0.03 M L-alanosine. The generation of $[^{14}C]O_2$ was assessed as follows: 5 µl of 40% KOH was added to the underside of the lids of the vessel prior to the addition of the enzyme, following which the vessels were quickly capped and incubated for the required time. The caps were then removed and radioactivity was measured by scintillation spectrometry. The following reaction mixture was constituted to examine the interaction of L-alanosine with this enzyme: 5 µl (0.25 microcurie) of DL-[3-^{14}C]-alanosine was admixed with 1 IU of L-aspartate-β-decarboxylase from *Alcaligenes faecalis* in 0.66 M sodium acetate, pH 4.8. The reactants were incubated at 37°C for 2 hrs and then subjected to high-voltage paper electrophoresis. The electropherogram channels were cut into 8-mm strips and radioactivity was measured by scintillation spectrometry.

11. *L-Aspartyl kinase (E. coli):* this enzyme was measured radiometrically by determining the rate of formation of L-[4-^{14}C]-aspartyl-β-hydroxamate in the presence of neutral hydroxylamine and ATP-$MgCl_2$. In 1600-µl polyethylene vessels, in a final volume of 20 µl, were admixed: 5 µl (0.25 microcurie) of L-[4-^{14}C]-aspartic acid; 5 µl of freshly neutralized hyroxylamine (0.04 M); 5 µl (0.2 µmol) of ATP-$MgCl_2$; and 5 µl of purified enzyme (1 mg/ml). The reagents were incubated at 37°C for 1 hr and then heated in open vessels at 95°C for 10 mins to destroy excess hydroxylamine. Residual L-aspartic acid was removed using the complete decarboxylation system described earlier. After 16 hrs at 25°C, 2 IU of L-asparaginase was added to the reaction mixtures at the same time as a 5-µl droplet of 40% KOH was deposited on the underside of the lid of the vessels; the lids were closed and the assemblies incubated at 37°C for 3 hrs, after which the lids were removed and any trapped $[^{14}C]O_2$ was measured by scintillation spectrometry. —continued

12. L-*Aspartate transport enzyme:* BDF_1 male mice were injected ip with 1×10^6 cells of L5178Y leukemia. 7 days later, tumor cells were aspirated from the peritoneum, collected by centrifugation at $1000 \times g$, washed twice with Dulbecco's phosphate buffered saline containing 2% glucose and 0.007% bovine serum albumin (fraction 5), and then suspended in the same medium to achieve a final concentration of 1×10^6 cells/ml. Transport studies were carried out at 37°C. To 2 ml of the above cell suspension, 0.05 ml of the labeled amino acid (0.15–10.8 nmols of L-[U-^{14}C]-aspartic acid) was added with constant agitation. At 0.3-min intervals, 900-μl aliquots of the cell suspension were layered over 700 μl of Versilube F-50 silicon oil in Eppendorf test tubes and immediately centrifuged at $12,000 \times g$ in an Eppendorf Zentrifuge for 1 min. The apex of the tube, containing the cell pellet, was cut off and the cell pellet was digested in 1.5 ml of 0.2 N NaOH and then counted by scintillation spectrometry. To study the effect of L-alanosine on the transport of these amino acids, similar incubation mixtures containing 1 or 10 mM L-alanosine were constituted; 2 ml of cell suspension was preincubated for 1 min with the designated concentration of L-alanosine in a volume of 50 μl, before the addition of labeled amino acid.

The only problem with this projection has emerged from our studies in vivo with two lines of murine leukemia resistant to L-alanosine developed by Randall Johnson (Arthur D. Little, Inc, Cambridge, Mass) and our studies in vitro with a line of neuroblastoma cells developed by F. Baskin and R. N. Rosenberg (University of Texas at Dallas). The enzymology of these lines is tabulated in table 2. It can be seen that the specific activity of adenylosuccinate synthetase, the target enzyme, is comparable in each of these variants, but that the specific activity of SAICAR synthetase is depressed in all three of the L-alanosine-resistant lines. In P388 leukemia, this depression is marked, suggesting that resistance in this line might stem from its inability to synthesize "alanosyl AICOR." Paradoxically, the activity of adenylosuccinate lyase is also depressed in all three resistant lines; since this enzyme most probably contributes to the decomposition of "alanosyl AICOR," it is difficult to reconcile lowered levels of it with resistance. This difficulty could be resolved if the fumaryl analog of L-alanosine, which is presumably produced by the action of this lyase on "alanosyl AICOR" (cf fig 6), were somehow involved in the therapeutic action of L-alanosine; however, to date, concrete evidence for this is lacking.

We are also beginning to examine the enzymes of purine salvage in these lines. This approach is all the more cogent in view of the fact that parenteral therapeutic doses of L-alanosine (400 mg/kg) only inhibit adenylosuccinic acid synthetase in tumor by 67%. With a faulty salvage system, this restriction in de novo biosynthesis of purines, while apparently of modest magnitude, just might tip the balance in the direction of starvation for adenine nucleotides.

Two aspects of L-alanosine pharmacology deserve emphasis: first, while some amino acids can function per se as inhibitors of target enzymes, others must undergo complex anabolism, or lethal synthesis, before the accomplishment of their therapeutic ends. Second, the *N*-hydroxy-*N*-nitroso functionality is a kind of surrogate

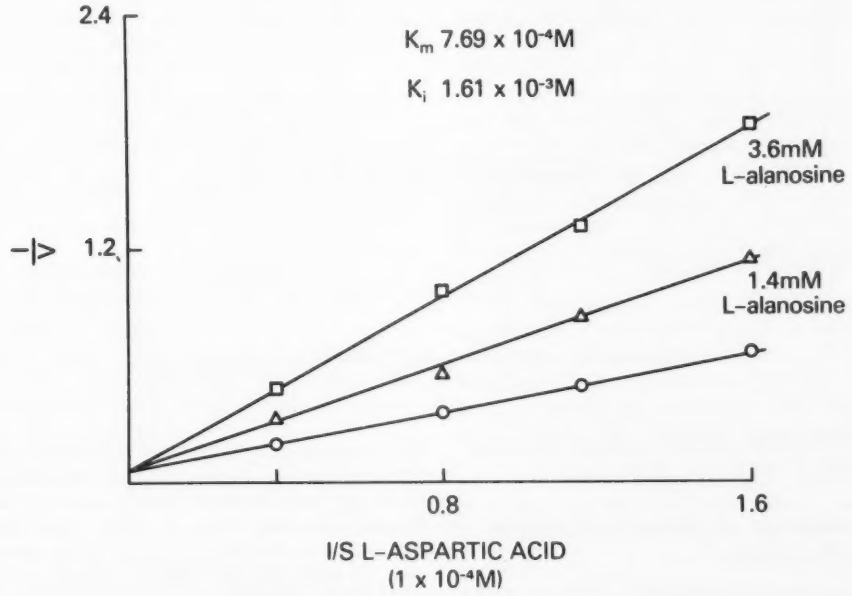

FIGURE 4.—Lineweaver-Burk plot of the inhibition by L-alanosine of L-aspartate utilization of SAICAR synthetase. SAICAR synthetase activity was measured according to the methodology detailed in table 1.

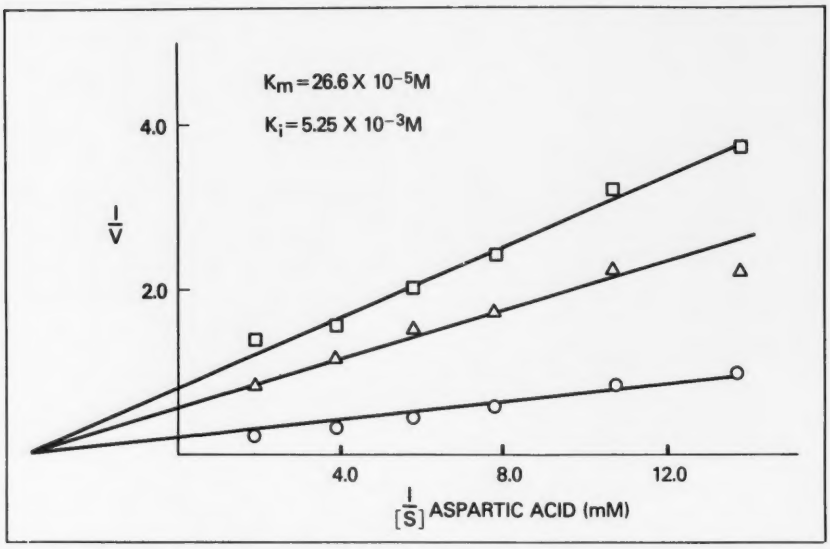

FIGURE 5.—Lineweaver-Burk plot of the inhibition of L-aspartate utilization of adenylosuccinate synthetase by L-alanosine. Adenylosuccinate synthetase activity was measured according to the method detailed in table 1. ○ = activity of control; △= activity in the presence of 3.6 mM L-alanosine; and □ = activity in the presence of 5.5 mM L-alanosine.

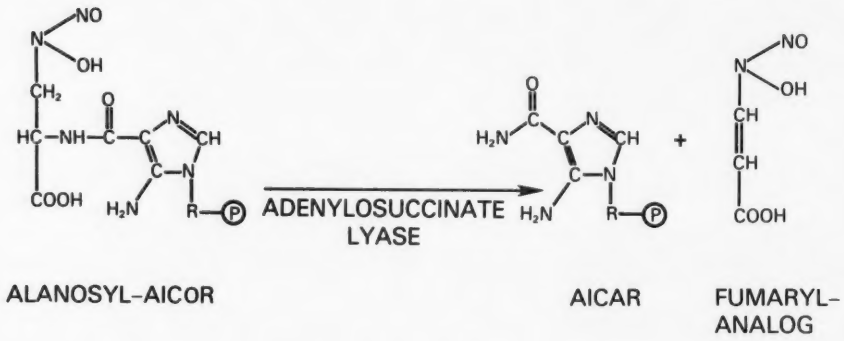

FIGURE 6.—Postulated decomposition of "alanosyl AICOR" by adenylosuccinate lyase.

carboxyl, and might be deployed by amino acid chemists as a reactive replacement for the carboxyl group.

Interaction of Analogs of L-Aspartic Acid With L-Asparagine Synthetase

We have also investigated the possibility that L-alanosine, by virtue of its homology to L-aspartic acid, might inhibit L-asparagine synthetase, another enzyme for which L-aspartic acid is the principal substrate. This expectation could not be verified experimentally, even when very high concentrations (up to 0.01 M) of the antibiotic were used. Thus, as table 1 also documents, L-alanosine is not a universal mimic of its prototype. However, at this point it would be appropriate to describe several other analogs of L-aspartic acid which do inhibit L-asparagine synthetase. Before doing so, the rationale which prompted this line of investigation should be reit-

TABLE 2.—Enzyme levels in L-alanosine-sensitive and -resistant lines

Enzymes*	P388 leukemia†		L1210 leukemia†		Neuroblastoma†	
	Sensitive	Resistant	Sensitive	Resistant	C46 (sensitive)	LA (resistant)
Adenylosuccinate synthetase	13.75 ± 6.05	11.10 ± 4.12	10.97 ± 3.99	9.50 ± 3.18	39.20 ± 8.80	24.11 ± 7.51
Adenylosuccinate lyase	185.70 ± 89.85	126.02 ± 66.39‡	115.92 ± 120.50	81.46 ± 42.49	439.09 ± 102.18	497.54 ± 181.49
SAICAR synthetase	2.71 ± 1.02	0.86 ± 0.38‡	1.40 ± 1.06	1.24 ± 0.64	1.77 ± 0.74	1.62 ± 0.58

*Adenylosuccinate synthetase activity was measured according to the procedure detailed in table 1. Adenylosuccinate lyase activity was measured in a reaction mixture of 15 μl containing 5 μl of [^{3}H]-adenylosuccinate acid (0.045 nmol, 103 nanocuries), 5 μl of adenylosuccinic acid (50 nmols), and 5 μl of tumor extract, prepared by homogenizing tumor nodules (1:4, wt/vol) in 0.1 M Tris-HCl, pH 7.6, containing 0.5 mM EDTA and 1.0 mM dithiothreitol. The reactants were mixed by centrifugation and incubated for 10 mins at 37°C. The reaction was terminated by heating at 95°C for 2 mins and the radioactive water was distilled overnight at room temperature into 10 μl of 100% KOH which was deposited on the lid. The lids were cut off and counted as described earlier. SAICAR synthetase activity was measured in a reaction volume of 20 μl, containing 5 μl of [^{14}C]-SAICAR (4.51 nmols, 29.04 nanocuries); 5 μl of sodium arsenate (50 nmols); 5 μl of ADP (50 nmols) or Tris-HCl buffer, pH 8.0 (250 nmols); and 5 μl of enzyme extract. The reactants were mixed by brief centrifugation and incubated at 37°C for 30 mins, and the reaction was terminated by quick freezing; 5 μl of 40% KOH was deposited on the lid at the same time that 50 μl of decarboxylation reagent (*see* adenylosuccinate synthetase) was dispensed to the apex of the vessels. The vessels were immediately closed and incubated at 37°C for 3 hrs, at which time the lids were cut off and counted as described earlier.

†Enzyme activities are expressed as nmols of product formed/mg of protein/hr.

‡$P < 0.005$.

erated. It is known from previous work that tumors resistant to L-asparaginase contain high levels of L-asparagine synthetase (9). It was reasoned therefore, that inhibition of this enzyme should render such tumors sensitive to L-asparaginase. The L-glutamine antagonists do inhibit L-asparagine synthetase, but their inhibition is not total (10). Moreover, this enzyme can use ammonia as a nitrogen donor even when the L-glutamine site is wholly alkylated and inactivated by these agents. Table 3 documents this point; of the six analogs of L-glutamine capable of interrupting the biosynthesis of L-asparagine, all are selective inhibitors of the utilization of the amide nitrogen of L-glutamine as opposed to free ammonia.[5]

Extrapolating from the efficiency of L-asparaginase in achieving very low levels of L-asparagine in plasma, total inhibition of L-asparagine synthetase presumably would be needed to reach a state of L-asparagine deprivation. In this regard, when the L-aspartic acid site of L-asparagine synthetase is occupied by a competitive inhibitor, use of both nitrogen donors is blocked. For this reason we thought that conjoint use of antagonists of L-glutamine and L-aspartic acid might more fully inhibit the functioning of this target enzyme.

Our previous studies with analogs of L-aspartic acid as inhibitors of L-asparagine synthetase from mammalian pancreas had suggested the following structural requirements for the L-aspartic acid site: the compound should be a small dicarboxylic acid in the L-configuration, where applicable, and with a free α-amino nitrogen. Some compounds which illustrate these principles are presented in table 4. Of the simple dicarboxylic acids, it can be seen that aminomalonic acid is rather potent, but that L-glutamic and α-amino adipic acids are inert as inhibitors. Thus, the carbon skeleton must equal that of L-aspartate or be shorter, but not longer.

The requirement for two carboxyl groups is demonstrated in 3-phosphono-L-alanine, L-cysteine sulfinic acid, L-cysteine sulfoximine, L-cysteine sulfoxide, L-alanosine, and β-cyano-L-alanine, all of which have β-substituents known to be sterically and electronically similar to a carboxylic acid, but all of which are poor inhibitors of the enzyme.[6]

Succinic acid, *N*-acetyl-L-aspartic acid, Umezawa's interesting new antibiotic, aziridinyl-L-aspartic acid, and L-malic acid are all without activity, suggesting, again, that a free α-amino function is required.[7]

Against this background it was surprising that the agents about to be discussed proved to be rather potent inhibitors of L-asparagine synthetase from tumor and pancreas.

The first of these is guanidinosuccinic acid, whose structure is presented in figure 7. It accumulates in human serum during uremic states to a concentration of 0.140 mM (11); at such concentrations guanidinosuccinate inhibits L-asparagine synthetase quite powerfully ($K_i \sim 0.9$ mM) (table 4). On structural grounds, straightforward

[5]Thiocyanoaminobutyric acid was synthesized and provided by Dr. Charlotte Ressler (University of Connecticut at Farmington). This compound selectively impedes the utilization of L-glutamine not only by L-asparagine synthetase, but also by PRPP amidotransferase and a panel of other amidotransferases. Thus, it is included in table 3 as a new antagonist of L-glutamine.

[6]The ability of the L-aspartyl dipeptides to inhibit L-asparagine synthetase may be attributable to their hydrolysis by peptidases contaminating the unpurified enzyme used as a source of L-asparagine synthetase. The resultant "cold" L-aspartic acid would dilute the specific activity of the radioactive L-aspartic acid used in the assay, giving rise to the appearance of inhibition. Transamination of oxaloacetate to L-aspartic acid could produce an identical effect.

[7]*N*-Formyl-aspartic acid, *N*-*O*-nitrophenylsulfenyl-L-aspartic acid, and β-aminoglutaric acid are exceptions to this rule.

TABLE 3.—Influence of L-glutamine antagonists in blocking ammonia utilization by L-asparagine synthetase*

Agent	Concentration (mM)	% inhibition of L-asparagine synthetase	
		Amido donor	
		L-Glutamine	Ammonia
6-Diazo-5-oxo-L-norleucine (DON)	1	78	0
5-Chloro-4-oxo-L-norvaline (CONV)	1	80	0
Chloroisoxazole [L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid]	1	85	0
L-(αS,4S,5R)-α-amino-3-chloro-4-hydroxy-4,5-dihydro-5-isoxazoleacetic acid	1	79	0
γ-Thiocyano-α-aminobutyric acid	1	75	0
Azotomycin	2	78	8
Albizzin	1	23	17
Duazomycin A	2	20	20
Azaserine	2	15	8
5-Hydroxylysine	2	0	0
γ-Glutamyl hydrazide	1	0	0

*L-Asparagine synthetase activity was measured according to the methodology detailed in table 1. The specific activity of the uninhibited tumoral enzyme was approximately 40 nmols of L-asparagine synthesized/mg of protein/hr.

competitive kinetics with L-aspartic acid as a variable substrate had been anticipated, but such was not the case: Lineweaver-Burk plots indicated noncompetitive inhibition. Although this result suggests that the interaction of guanidinosuccinic acid with L-asparagine synthetase is not as straightforward as we supposed, the main point to be made with respect to this agent is that it illustrates the possibility of substitutions on the α-amino nitrogen, with retention of potency. It seems likely that the basicity which amino and guanidino substituents have in common is responsible for this retention.

The second inhibitor of L-asparagine synthetase, PA_2LA [3-(phosphonacetylamido)-L-alanine] (fig 8), violates both of the structural rules stated earlier. Not only is it longer in space than L-aspartic acid, but it also lacks the β-carboxyl group. Instead, a phosphonate assumes the role of the distal substituent. PA_2LA was synthesized by Frederick Wedler (Rensselaer Polytechnic Institute, Troy, NY) as an inhibitor of bacterial and mammalian L-glutamine synthetase. Versus L-asparagine synthetase this compound is also a moderately potent noncompetitive inhibitor (fig 9); our preliminary results also indicate that it is capable of inhibiting L-aspartate aminotransferase (data not shown). These findings warrant its being labeled an analog of L-aspartic acid. Moreover, on the basis of this behavior, it is possible to add the phosphonate group to the list of what have been called "surrogate carboxyls."

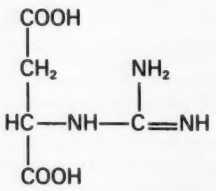

FIGURE 7.—Chemical structure of guanidinosuccinic acid.

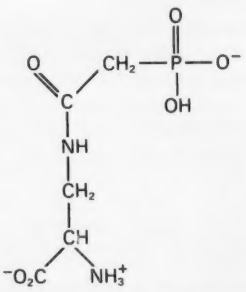

FIGURE 8.—Chemical structure of PA_2LA [3-(phosphonacetylamido)-L-alanine].

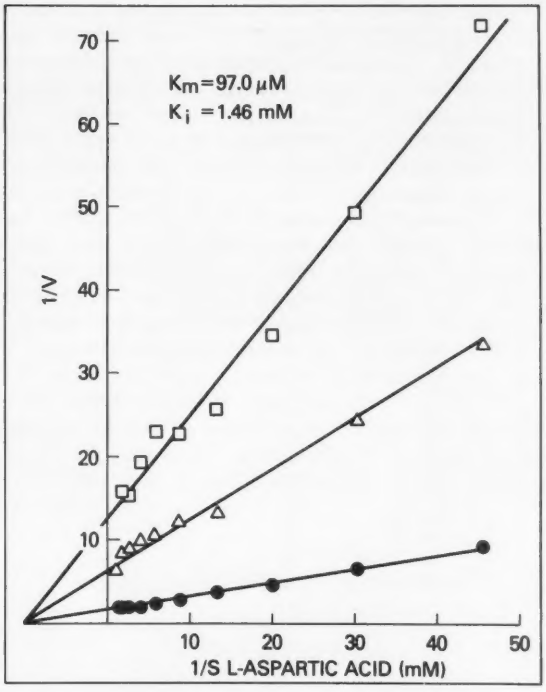

FIGURE 9.—Lineweaver-Burk plot of the inhibition of L-asparagine synthetase by PA_2LA. L-Asparagine synthetase activity was measured as detailed in table 1. ○ = activity of control; △= activity in the presence of 2.0 mM PA_2LA; and □ = activity in the presence of 4.0 mM PA_2LA.

The third agent in this brief compilation is L-homoserine-β-adenylate.[8] This nucleotide is a homolog of L-aspartic acid-β-adenylate, the presumed transitory intermediate formed when L-aspartic acid and ATP interact at the catalytic center of L-asparagine synthetase. In our hands, it has proven to be a very potent inhibitor of this enzyme from murine pancreas (table 4), engendering inhibition which is reversible but kinetically complex; comparatively high concentrations of magnesium are required for inhibition to be expressed by L-homoserine-β-adenylate, and the inhibition is not competitive with either L-aspartic acid or with ATP (K_i~ 0.2 μM) (12). Unfortunately, the phosphate group probably excludes L-homoserine-β-adenylate from the intracellular space; thus, it has been demonstrated that the compound is without cytotoxicity to L-asparagine-dependent lymphoblasts in culture, at concentrations ranging from 1 to 100 μM.

For this reason, the nucleoside analog of homoserine-β-adenylate is being synthesized in the hope that it will traverse the cell membrane and be phosphorylated intracellularly, by analogy to a number of other antimetabolites.

L-Aspartic Acid Transcarbamylase as a Target for Chemotherapy

PALA (*N*-phosphonacetyl-L-aspartic acid) is a transition-state analog of another type. This compound specifically inhibits L-aspartate transcarbamylase but not other L-aspartate-related enzymes. For example, it wholly fails to inhibit L-aspartic acid-β-decarboxylase, L-asparagine synthetase, L-aspartic acid aminotransferase, adenylosuccinate synthetase, or SAICAR synthetase, presumably because of the bulky, negative substituent projecting from its α-amino nitrogen. However, precisely because of this configuration, it potently inhibits L-aspartic acid transcarbamylase, an enzyme which catalyzes the formation of an analogous transition-state intermediate from L-aspartic acid and carbamyl phosphate in the course of the biosynthesis of carbamyl-L-aspartic acid (fig 10) (13). By virtue of its interaction with L-aspartic acid transcarbamylase, PALA interrupts the biosynthesis of the pyrimidine ring, and by virtue of this interruption, the drug has proven to be a potent antitumor agent, with

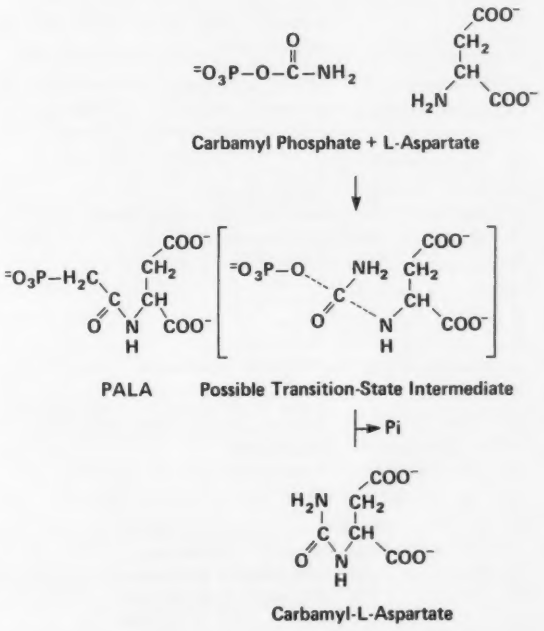

FIGURE 10.—Reaction catalyzed by L-aspartate transcarbamylase and the relationship of PALA (*N*-phosphonacetyl-L-aspartic acid) to the transition-state intermediate.

[8] L-Homoserine-β-adenylate was synthesized and kindly provided by Drs. P. Chang, R. E. Handschumacher, and J. Uren (Yale University, New Haven, Conn).

TABLE 4.—Inhibition of L-asparagine synthetase by analogs of L-aspartic acid*

Analog	% inhibition of L-asparagine synthetase	
	Amido donor	
	L-Glutamine	Ammonia
Homoserine-β-adenylate	95	90
Guanidinosuccinic acid	81	80
Oxaloacetic acid	56	55
N-*O*-Nitrophenylsulfenyl-L-aspartic acid	51	44
L-Aspartyl-L-phenylalanine	45	30
L-Aspartyl-L-valine	44	53
3-Hydroxy-DL-erythro-aspartic acid	44	53
PA_2LA [3-(phosphonacetylamido)-L-alanine]	44	45
meso-α,β-Diaminosuccinic acid	43	45
N-Formyl-L-aspartic acid	41	47
Aminomalonic acid	41	37
β-Aminoglutaric acid	40	41
O-Phospho-L-serine	35	36
L-Aspartyl-L-serine	34	48
L-Cysteine sulfinic acid	28	20
L-Aspartyl-L-proline	27	41
L-Aspartyl-L-leucine	25	26
3-Phosphonoalanine	9	0
N-Acetyl-L-glutamic acid	6	0
N-Acetyl-L-aspartic acid	0	0
L-Alanosine	0	0
L-Allo-α-hydroxyglutamic acid	0	0
DL-α-Aminoadipic acid	0	0
2-Amino-4-butyric acid	0	0
Aziridinyl-L-aspartic acid	0	0
N-Carbamyl-DL-aspartic acid	0	0
S-Carbamyl-L-cysteine	0	0
β-Cyano-L-alanine	0	0
L-Cysteic acid	0	0
DL-Cysteic acid	0	0
L-Cysteine sulfoximine	0	0
L-Cysteine sulfoxide	0	0
2,4-Diamino-glutaric acid	0	0
DL-2,3-Diaminosuccinic acid	0	0
Fumaronitrile	0	0
L-Glutamic acid	0	0
L-Glutamic acid, 5-methyl ester	0	0
L-Homoserine	0	0
4-Hydroxy-DL-glutamic acid	0	0
3-Hydroxy-DL-threo-aspartic acid	0	0
Ketomalonic acid	0	0
L-Malic acid	0	0
Malonic acid	0	0
3-Methyl-DL-aspartic acid	0	0
D-α-Methylglutamic acid	0	0
PALA (*N*-phosphonacetyl-L-aspartic acid)	0	0
N-Phosphonacetyl-L-glutamic acid	0	0
Succinic acid	0	0
Tartaric acid	0	0
DL-Threonine	0	0

*L-Asparagine synthetase activity was measured according to the methodology detailed in table 1. Inhibitors were added to a final concentration of 1 mM for these studies. Further information on the compound designated "aziridinyl-L-aspartic acid" (*S*-2,3-dicarboxyaziridine) can be found in J Antibiot 28:828–829, 1975. We are grateful to Prof. Umezawa for his gift of a sample of this substance.

outstanding activity against many rodent solid carcinomas, including several which had been rather consistently refractory to conventional chemotherapy (14). It is noteworthy, from the standpoint of predictive enzymology, that these susceptible tumors have, in general, significantly lower levels of L-aspartic acid transcarbamylase than resistant lines (fig 11). However, the distinctions are not as marked as in the case of L-asparagine synthetase, where certain L-asparaginase-sensitive neoplasms wholly lack L-asparagine synthetase. George Stark's group (Stanford University, Calif) and Randall Johnson (formerly at the National Cancer Institute, Bethesda, Md) made analogous observations on the specific activity of L-aspartic acid transcarbamylase in cultured tumor cells (15,16). In vitro, a doubling of the specific activity of the target enzyme is accompanied by a tenfold elevation of the median lethal dose of PALA. This finding documents that minor modulations of L-aspartic acid transcarbamylase can have profound therapeutic consequences.

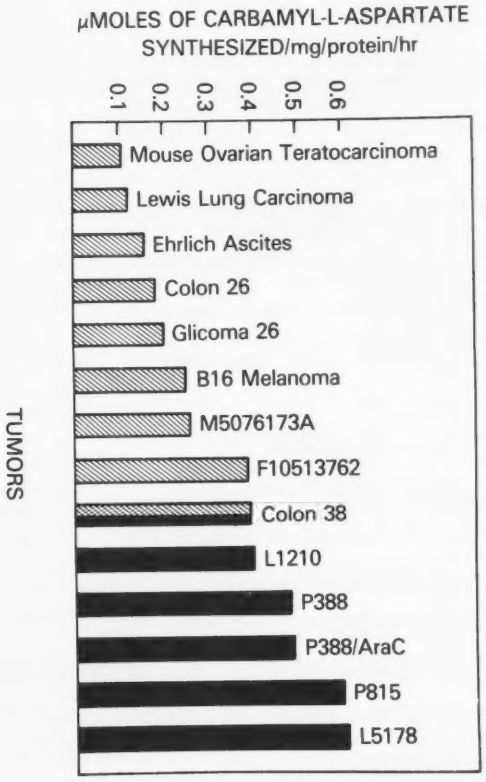

FIGURE 11.—Specific activity of L-aspartate transcarbamylase in tumors sensitive and resistant to PALA. ▨ = tumors sensitive to PALA; ▩ = tumors partially sensitive to PALA; and ■ = tumors resistant to PALA.

For all of these reasons, PALA has entered clinical trials this year with a good deal of anticipation. Preparatory to these trials, two puzzling features of the mechanism of action of PALA have been encountered. The first such puzzle arose in the course of studies which had as their aim the development of an analytic method for the measurement of PALA in biologic fluids (17). The technique was to be based on an enzyme-inhibition assay. We had on hand for this purpose a purified preparation of L-aspartic acid transcarbamylase from *Escherichia coli* (18), donated by E. Kantrowitz (Harvard University, Boston, Mass), and a crude splenic enzyme prepared according to Tatibana and Ito (19) from mouse spleen. Against the mammalian enzyme, PALA was as potent as the literature indicated ($K_i \sim 0.7$ μM); against the bacterial enzyme, it was not ($K_i \sim 800$ μM). This disparity appeared to contradict published reports suggesting that PALA was roughly equipotent towards both enzymes. The discrepancy was heightened when we observed that certain concentrations of PALA (5×10^{-6} M to 5×10^{-5} M) even stimulated our preparation of L-aspartic acid transcarbamylase from *E. coli*, and did so with notable potency. The negative then positive response of this enzyme to PALA is illustrated in figure 12.

Exactly why these differences have been observed is not clear, although it is relevant that higher concentrations of carbamyl phosphate and lower concentrations of L-aspartic acid were used for these studies than in those of most other workers in this area.

The second intriguing aspect of PALA's mechanism of action is the pertinacity with which it inhibits L-aspartate transcarbamylase in vivo. This is documented for spleen and tumor in figure 13. After a single dose of 400 mg/kg, restitution of enzyme activity is slow, amounting only to 50% of control at 8 days in spleen and tumor. In liver, after a single such dose, the restitution of enzyme activity is only 11.8%, 12.3%, 29%, and 30.0% of control after 6, 12, 16, and 20 days respectively. Since PALA is a potent inhibitor of its target enzyme in mammals, one might expect tight binding initially. However, this binding is reversible in vitro and is known to be fully competitive with carbamyl phosphate. In vitro, one can even dislodge PALA from the active site with carbamyl phosphate. If this same dislodgment were to occur in vivo, then activity would be restored far more rapidly than found in this study. The resolution of this puzzle may reside in the fact that carbamyl phosphate is compartmentalized in vivo, with the major portion formed, and used, within the mitochondrion; this pool would be inaccessible to L-aspartic acid transcarbamylase in the cytosol and could not function to compete with PALA.

On the basis of the long-lasting inhibition produced by PALA of L-aspartic acid transcarbamylase in liver, it can also be speculated that the rate of biosynthesis of new enzyme molecules in that organ is slow or, alternatively,

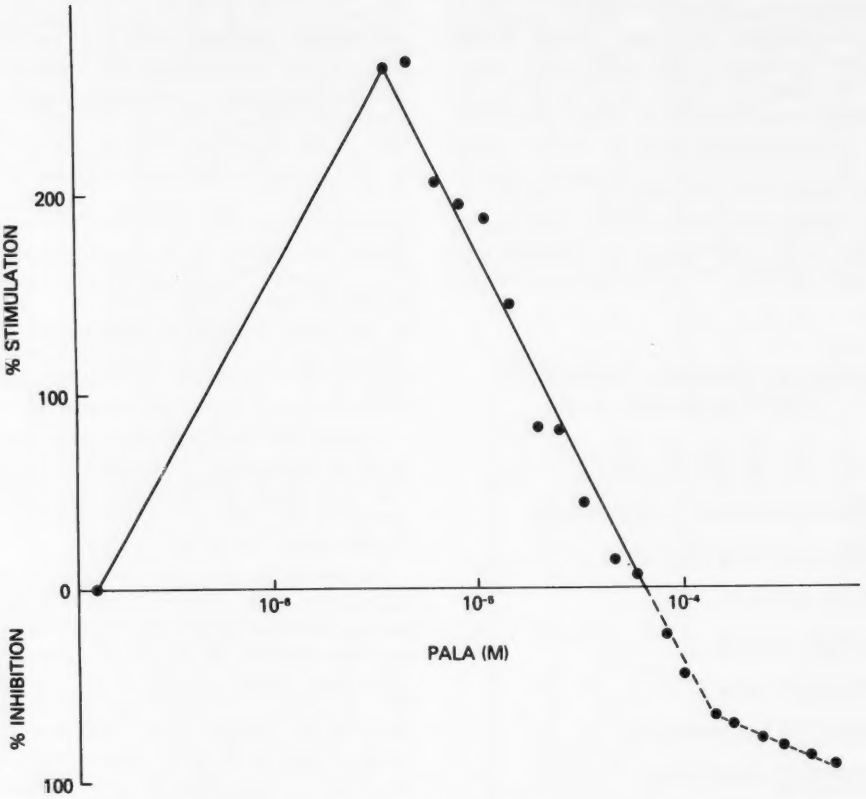

FIGURE 12.—Influence of various concentrations of PALA on L-aspartate transcarbamylase from *E. coli*. L-Aspartate transcarbamylase activity was measured as detailed in table 1.

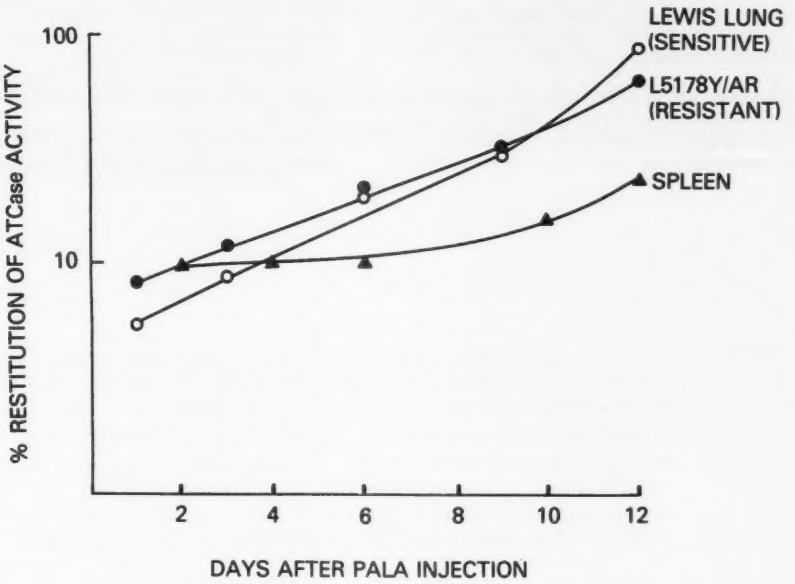

FIGURE 13.—Time course of restitution of L-aspartate transcarbamylase levels in mouse tissues after a single therapeutic dose of PALA (400 mg/kg, ip). L-Aspartate transcarbamylase activity was measured according to the methodology detailed in table 1.

that there is efficient shuttling of inhibitor from old to new molecules as they are synthesized.

In summation, this report presents a rather broad and miscellaneous group of agents. Of the molecules discussed, most are natural products, but some are synthetic. On the basis of these data, we suggest that L-aspartic acid is a rightful point of focus for the synthesis of new analogs.

ADDENDUM

Since the submission of this manuscript, it has been brought to the authors' attention by F. Wedler and E. Kantrowitz that Collins and Stark (13) have also reported the stimulation of L-aspartic acid transcarbamylase from *E. coli* by PALA.

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Enhanced Effect of an L-Glutamine Antagonist, L-(αS,5S)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, by *Acinetobacter* L-Glutaminase-L-Asparaginase [1]

J. S. Holcenberg [2,3,*]

SUMMARY

The effect of L-glutamine and L-asparagine depletion by *Acinetobacter* L-glutaminase-L-asparaginase on the toxicity and antitumor activity of L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (NSC-163501) was tested in mice. The LD50 of six daily doses of NSC-163501 in BDF_1 female mice decreased from 7.5 to 0.3 mg/kg/day by combination treatment with the enzyme. Enzyme therapy also decreased the dose of NSC-163501 needed for maximal prolongation of survival in these mice inoculated with L1210 leukemia. Nevertheless, the combination did not prolong survival in L1210-bearing mice beyond that of higher doses of NSC-163501 alone. In contrast, the combination of enzyme plus NSC-163501 inhibited the growth of established sc implanted Ehrlich ascites carcinoma in ICRf male mice much more than either agent alone. Treatment with *Acinetobacter* L-glutaminase-L-asparaginase decreased the L-asparagine and L-glutamine levels in acid extracts of the Ehrlich tumor. NSC-163501 did not affect the amide levels or alter the decrease produced by enzyme therapy.

[Cancer Treat Rep 63:1109–1114, 1979]

L-(αS,5S)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (NSC-163501) is a potent inhibitor of L-asparagine synthetase and other enzymes that catalyze amido group transfer from L-glutamine (1,2). This drug has antitumor activity against L1210 and P388 mouse leukemias, a mouse ovarian tumor, and xenografts of a human mammary tumor and an oat cell carcinoma in nude mice (3–5). The growth inhibition of NSC-163501 on L1210 cells in tissue culture can be reversed by the addition of L-glutamine to the medium (2,6). Similarly, L-glutamine partially blocks the inhibition of L-asparagine synthetase by the drug (1). Therefore, reduction of plasma levels of L-glutamine might potentiate the activity of NSC-163501 in vivo. For combination chemotherapy, *Acinetobacter* L-glutaminase-L-asparaginase could be used to deplete L-glutamine levels since this enzyme is not inhibited by NSC-163501 (7). This paper reports the antitumor and toxic effects of combination therapy of mice with *Acinetobacter* L-glutaminase-L-asparaginase and NSC-163501.

MATERIALS AND METHODS

Animals.—L1210 leukemia was grown in 20–30-g $(C57BL \times DBA/2)F_1$ (BDF_1) female mice (Charles River Breeding Laboratories, Wilmington, Mass). Ehrlich ascites carcinoma was grown in 20–30-g male SCH:ARS outbred Swiss albino mice (ICRf) (Sprague-Dawley, Madison, Wisc). Mice were maintained in groups of five per filter top cage and were fed Wayne Lab Blox (Allied Mills Inc, Chicago, Ill) or Purina mouse chow and water ad libitum. All mice were infected with lactate dehydrogenase-elevating virus by ip injection of 0.1 ml of a one tenth dilution of plasma from a previously infected mouse. This virus infection prolongs the half-life of the *Acinetobacter* L-glutaminase-L-asparaginase (8).

[1]Supported in part by Public Health Service grants CA-20061 and CA-17700 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare, and by the Faye McBeath Foundation. Dr. Holcenberg is a Burroughs Wellcome Scholar in Clinical Pharmacology.

[2]Departments of Pediatrics and Pharmacology, the Medical College of Wisconsin, and Midwest Children's Cancer Center, Milwaukee Children's Hospital.

[3]I wish to thank B. Ring, E. Tang, and P. Lu for excellent technical assistance and G. Neil and L. J. Hańka for helpful discussions.

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Tumors.—L1210 leukemia (generation B18, strain LE 18) was originally obtained from Microbiological Associates (Bethesda, Md) in 1972. It was propagated in our laboratories by ip inoculation in DBA/2 (Charles River Breeding Laboratories) and BDF_1 female mice and was stored frozen in 15% glycerol. The Ehrlich ascites carcinoma (SK line) (9) was propagated in our laboratories by ip inoculation in female and male ICRf mice and was stored frozen in 15% glycerol.

Drug.—NSC-163501 was obtained from Drs. J. Douros and H. Wood, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md), through Microbiological Associates and from Dr. L. Haňka, The Upjohn Company (Kalamazoo, Mich) (lot Nos. 10,985 DGM146.20 and DGM153.16). Material from both lots showed only one spot reacting with Ninhydrin after chromatography on cellulose thin-layer strips (Eastman No. 6065) in *N*-butanol:acetic acid:water (80:20:20), carbon tetrachloride:methanol:ammonium hydroxide (40:40:20), and 90% ethanol. The drug appeared to be stable in saline solution for at least 1 week at 5°C.

Enzyme.—Acinetobacter L-glutaminase-L-asparaginase was purified to homogeneity as previously reported (10,11) and was stored as a lyophilized powder at 5°C. All lots had a specific activity of > 120 IU/mg of protein. Solutions in saline were stable for at least 1 week at 5°C. Enzyme was assayed in plasma by ammonia release from L-asparagine (10). Amino acid analysis was performed on sulfosalicylic acid extracts of plasma and tissues on a JOEL 5AH amino acid analyzer with 0.3 M lithium citrate buffer, pH 2.88 (11). In some tissue samples the high L-glutamate peak was not completely separated from the L-asparagine and L-glutamine peaks. A 2-ml portion of these tissue extracts was neutralized with LiOH and chromatographed on 0.4 × 6-cm Pasteur pipette columns of AG1 X 8 (200–400 mesh) previously washed and neutralized in 10 mM imidazole buffer, pH 7. The columns were washed with 4 ml of the same buffer. The combined eluates were lyophilized, dissolved in lithium citrate buffer, and chromatographed on the analyzer. This treatment removed most of the L-glutamate and L-aspartate but did not alter the levels of the other amino acids.

Treatments.—Mice were injected ip with saline, enzyme, or combinations in a volume of 0.01 ml/g. For the L1210 experiments, BDF_1 mice were injected ip with 10^5 L1210 cells on Day 0. Mice were randomly assigned to groups and treatment was given ip on Days 1–7. All dead mice were autopsied to measure ascites fluid, tumor size, and spleen weight. For experiments with the Ehrlich ascites carcinoma, 10^6 tumor cells were transplanted sc into ICRf mice on Day 0. The tumor was palpable after 5–7 days. At that time the mice were assigned to groups with equal average tumor size and treatment regimens were assigned randomly. Mice were killed by ether anesthesia and exsanguination 1 day after the sixth daily treatment. Tumor and tissues were removed, blotted, weighed, homogenized in 10% sulfosalicylic acid, and analyzed for amino acid levels (11). Plasma was assayed for enzyme activity and was deproteinized for amino acid analysis.

RESULTS

Toxic Effects

BDF_1 female mice infected with lactate dehydrogenase-elevating virus were treated with six consecutive daily ip doses of 0–32 mg/kg of NSC-163501 alone or in combination with six daily doses of 150 IU/kg of *Acinetobacter* L-glutaminase-L-asparaginase. Only one of nine mice died after treatment with 2 mg/kg/day of NSC-163501 while all mice died after treatment with 0.5 mg/kg/day of this drug plus enzyme. Analysis of the data by moving averages (12,13) gave an LD50 of 7.5 mg/kg/day × 6 days for NSC-163501 alone and an LD50 of 0.3 mg/kg/day × 6 days for the combination treatment.

Body weight changes also showed an increased toxicity of the combination of enzyme and this drug. On Day 5 of treatment, mice treated with 1 mg/kg/day of NSC-163501 and enzyme had lost 20% of their body weight. In contrast, doses of drug alone of > 8 mg/kg/day were needed to produce the same weight loss. In a separate experiment, groups of mice without lactate dehydrogenase-elevating virus were treated with the same dose of enzyme plus 0.1–0.8 mg/kg/day of drug × 6 days. None of these mice lost weight or died.

Table 1 shows the levels of acid-soluble amino acids in extracts of plasma, brain, liver, spleen, and kidney. These samples were obtained 24 hours after three daily ip injections of saline, 150 IU/kg of enzyme, 1 mg of drug, or a combination of drug and enzyme in BDF_1 female mice (two mice per group). Average plasma L-glutaminase activity was 0.60 IU/ml in mice treated with the enzyme and 0.45 IU/ml in mice treated with the combination. The enzyme depleted plasma levels of L-asparagine and L-glutamine to < 0.005 mM and increased L-aspartate and L-glutamate levels to 0.1 and 2 mM, respectively. The drug did not appreciably change plasma levels of these amino acids in comparison with control mice or alter the effect of the enzyme on these amino acids.

Enzyme treatment lowered the levels of L-glutamine in spleen extracts. This effect was less pronounced in mice treated with enzyme plus drug. As reported previously (11), brain levels of L-glutamine rose during enzyme therapy. Brain L-glutamine levels were even higher in mice treated with enzyme and drug. Kidney L-glutamate levels rose only during enzyme therapy without drug. Liver extracts showed increases in L-asparagine,

TABLE 1.—Effect of treatment on amino acid levels in BDF_1 female mice*

Tissue	Drug dose (mg/kg/day × 3)	Amino acid concentration†							
		L-Aspartate	L-Asparagine	L-Glutamate	L-Glutamine	L-Aspartate	L-Asparagine	L-Glutamate	L-Glutamine
		Without enzyme				*With enzyme*			
Plasma	0	0.020	0.035	0.067	0.505	0.132	< 0.005	2.13	< 0.005
	1	0.038	0.033	0.101	0.560	0.130	< 0.005	1.91	< 0.005
Spleen	0	3.40	0.32	7.87	1.26	3.05	0.09	5.80	0.40 ‡
	1	3.40	0.33	8.29	1.36	2.82	0.10	6.14	0.76
Brain	0	2.08	0.09	10.55	4.26	2.16	0.12	10.43	6.44
	1	2.31	0.10	10.83	4.92	1.76	0.08	10.48	9.33
Kidney	0	2.00	0.11	4.83	0.97	3.88	0.05	10.46	2.99
	1	1.34	0.15	4.62	1.47	2.76	0.03	5.33	1.53
Liver	0	1.90	0.16	0.97	2.85	0.58	0.15	1.16	3.30
	1	1.14	0.49	1.76	5.30	1.02	0.27	1.72	3.69

*Amino acid levels were assayed 24 hrs after 3 daily doses of drug (NSC-163501), enzyme (150 IU/kg of *Acinetobacter* L-glutaminase-L-asparaginase), or both.

†Average μmols/g of wet weight of tissue of amino acids in sulfosalicylic acid extracts from 2 mice/treatment.

‡Boxed values represent consistent, major differences.

TABLE 2.—Effect of drug and enzyme on ip L1210 leukemia*

NSC-163501 (mg/kg)	Enzyme†	No. of mice dying with tumor	Mean survival time (days ± 1 SE)‡	% increase in lifespan over controls	Early deaths§
—	–	18	6.6 ± 0.3	—	
—	+	9	8.2 ± 0.1	24	
1	–	5	9.0 ± 0.0	36	
2	–	5	11.2 ± 0.8	70	
4	–	8	14.2 ± 0.3	115	1/9
0.25	+	7	10.6 ± 0.3	61	1/8
0.5	+	7	12.6 ± 0.4	92	1/8
1.0	+	6	12.7 ± 0.6	92	3/9

*10^5 L1210 cells transplanted ip in BDF_1 mice on Day 0; drug and/or enzyme given ip on Days 1–7. Controls received saline.

†150 IU/kg of *Acinetobacter* L-glutaminase-L-asparaginase; – = no enzyme treatment and + = enzyme treatment.

‡All treatments were significantly different from saline controls by the Student's t test ($P < 0.05$). All treatments, except enzyme alone, were significantly different from saline controls by 1-way analysis of variance ($P < 0.05$).

§No. of mice dying before Day 9 and having no splenic enlargement or ascites/total No. of mice.

L-glutamate, and L-glutamine levels after drug treatment, as well as small changes in L-asparagine and L-glutamine after treatment with drug plus enzyme. None of these tissues or plasma extracts showed changes in L-serine or L-threonine levels (2).

Antitumor Effects

Effect on L1210 leukemia.—Table 2 presents the results of treatment of BDF_1 female mice injected with 10^5 L1210 leukemia cells. Treatment with enzyme alone produced only a small increase in survival. Drug treatment increased survival with significant improvement occurring as the dose was increased from 1 to 4 mg/kg/day ($P < 0.05$ by one-way analysis of variance). Combination treatment with 1 mg/kg/day of NSC-163501 plus enzyme improved survival time in comparison to either this dose of drug or the enzyme alone. Enzyme plus drug at 2 or 4 mg/kg/day produced severe weight loss and almost all of the mice died before Day 9, with no signs of leukemia. The survival time for enzyme plus 1 mg/kg/day of drug was significantly less than that of the highest dose of drug alone. Thus, the combination chemotherapy appeared to increase antitumor activity and toxicity to a similar degree.

Effect on Ehrlich ascites tumors.—*Acinetobacter* L-

TABLE 3.—Effect of drug and enzyme on sc Ehrlich ascites carcinoma*

NSC-163501 (mg/kg)	Enzyme†	No. of mice dying with tumor	Tumor weight (%)		Carcass weight (%)	
			Median	Mean ± SE	Median	Mean ± SE
0.5	–	5	103	120 ± 15	106	106 ± 4
1	–	10	77	82 ± 13	103	102 ± 2
2	–	5	107	100 ± 19	97	99 ± 3
4	–	10	63	71 ± 12	99	95 ± 3
8	–	10	44	62 ± 13	86	88 ± 3
16	–	8‡	55	70 ± 15	88	86 ± 2
—	+	15	105	113 ± 15	83	85 ± 4
0.25	+	10	92	84 ± 13	84	84 ± 5
0.5	+	14§	37	36 ± 6	77	77 ± 4
1	+	15	19	22 ± 3	64	69 ± 5
2	+	10	20	19 ± 4	65	71 ± 5
Pair-fed¶		9§	62	56 ± 8	64	67 ± 4

*Results of 4 experiments with 10^6 Ehrlich ascites cells injected sc into ICRf male mice on Day 0. Treatment was given ip daily for 6 days beginning on Days 5–7. Mice were killed 24 hrs after the last treatment. Tumor weights ± SE for saline-treated mice = 4.8 ± 1.0, 2.8 ± 2.0, 4.0 ± 1.1, and 2.6 ± 1.3 g in the experiments (No. = 5 for each group). Results are expressed as % of saline solution.

†300 IU/kg of *Acinetobacter* L-glutaminase-L-asparaginase; – = no enzyme treatment and + = enzyme treatment.

‡2 additional mice died of toxicity.

§1 additional mouse died of toxicity.

¶Pair-fed with group receiving enzyme plus 1 mg/kg of drug. 1 mouse died of infection.

glutaminase-L-asparaginase is effective therapy for Ehrlich ascites carcinoma and several other mouse tumors grown in the ascitic form (14,15). The enzyme has much less effect on the same tumors grown subcutaneously (15). In the subcutaneous tumors, the connective tissue may provide some L-glutamine to the tumor cells or limit the effective concentration of enzyme in the fluid surrounding the tumor. L-Glutamine antagonists might increase the antitumor activity of the enzyme by decreasing the production or utilization of L-glutamine. Therefore, mice received Ehrlich carcinoma cells transplanted sc and were treated with enzyme, drug, or both for 6 days beginning 5–7 days after tumor transplantation.

Table 3 shows the effect of treatment on tumor and carcass weight. As the dose of drug increased from 0.5 to 16 mg/kg/day × 6, the tumor weight decreased to a plateau level of 62%–71% of the saline-treated controls. Carcass weight decreased only to 86% of the control. Treatment with enzyme alone did not decrease tumor weight but lowered carcass weight to 85% of the saline control. The combination of enzyme plus drug had a marked synergistic effect on tumor weight. Tumor weight was 22% and 19% of the saline control after six daily treatments with enzyme plus 1 or 2 mg/kg of drug. These doses of drug alone did not decrease tumor weight appreciably. Carcass weight decreased 30% in mice treated with enzyme and 1 or 2 mg/kg of drug. A group of mice was treated with saline and pair-fed with the groups treated with enzyme plus 1 mg/kg of drug. This starvation regimen produced a similar decrease in tumor and carcass weight (44% and 33%, respectively).

At the time the mice were killed, the average plasma L-glutaminase activity in groups treated with enzyme alone and enzyme plus 2 mg/kg/day of drug was 0.4 and 0.2 IU/ml, respectively. Plasma L-glutamate was 1.92 and 1.20 mM in these groups. Treatment with 2 mg/kg/day did not affect the levels of L-aspartate, L-asparagine, L-glutamate, L-glutamine, L-serine, L-threonine, or L-glycine in plasma or liver extracts. Table 4 shows the amino acid levels in extracts from tumor tissue. Enzyme treatment decreased the tumor L-asparagine and L-glutamine levels to 20% and 50% of control levels, and increased the L-threonine, L-serine, and L-glycine levels. Levels of these amino acids in extracts from mice treated with drug alone did not differ from controls. Furthermore, extracts from the group treated with the enzyme plus drug had essentially the same levels as enzyme alone.

DISCUSSION

L-Glutamine antagonists and analogs have not proven to be clinically useful antitumor agents. As discussed in the proceedings in this issue, part of this failure may be due to suboptimal dose schedules. The nonspecific inhibition of L-glutamine-utilizing enzymes by drugs such as 6-diazo-5-oxo-L-norleucine may also contribute to the failures (16). This drug inhibits *Acinetobacter* L-glutaminase-L-asparaginase activity as well as many amidotransferases and thereby minimizes any potential combined effect of the two antitumor agents (15).

NSC-163501 is a more selective L-glutamine antagonist

TABLE 4.—Effect of treatment on amino acid levels in sc Ehrlich ascites carcinoma*

NSC-163501 (mg/kg/day × 6)	Enzyme†	Amino acid concentration‡						
		L-Aspartate	L-Asparagine	L-Glutamate	L-Glutamine	L-Threonine	L-Serine	L-Glycine
0	–	1.16	0.21	2.27	0.75	1.05	0.44	2.62
0	+	0.93	0.04	2.50	0.38	1.37	0.66	5.52
2	–	0.94	0.17	2.14	1.01	0.83	0.39	2.57
2	+	0.90	0.05	2.37	0.49	1.58	0.62	4.96

*Amino acids were assayed in sulfosalicylic acid extracts of tumor from the experiment summarized in table 3.
†300 IU/kg of *Acinetobacter* L-glutaminase-L-asparaginase; – = no enzyme treatment and + = enzyme treatment.
‡Average μmols/g of wet weight of amino acids in extracts from 2 mice/treatment.

that strongly inhibits L-asparagine synthetase and certain other amidotransferases but does not inhibit L-glutamine synthetase from *Escherichia coli* or rat liver, 5-phosphoribosylamine synthetase from fetal liver, *E. coli* (pH 5) L-glutaminase (2), or *Acinetobacter* L-glutaminase-L-asparaginase. The experiments reported in this paper show that daily doses of this drug in combination with L-glutaminase-L-asparaginase have greater host toxicity and antitumor activity in mice transplanted with both L1210 leukemia and Ehrlich ascites carcinoma than NSC-163501 alone. With L1210 leukemia, the toxicity and antitumor activity balance so that the combination produces no improvement in survival.

In contrast, the combination produces a much greater effect on sc transplanted Ehrlich ascites carcinoma than either agent alone. Levels of L-glutamine and L-asparagine in acid-soluble extracts of plasma and residual tumors were not altered by addition of drug to the enzyme therapy. The level of these amino acids should be lowered if NSC-163501 decreased the ability of the connective tissue to supply the tumor cells with L-glutamine or L-asparagine. Possible mechanisms for this synergism include increased cellular uptake or retention of the drug, alteration of specific intracellular pools of amino acids, and decreased synthesis of target enzymes. Elucidation of the mechanism will require sensitive methods to measure NSC-163501 and further studies of its inhibition of specific amidotransferases.

The effect of drug and drug plus enzyme on acid-soluble amino acid levels appears to vary with tissue, treatment schedule, and mouse species. For instance, the levels of L-asparagine, L-glutamate, and L-glutamine change in liver of BDF_1 female mice and ICRf female mice (11) after three daily treatments, but these levels did not change after six daily treatments in ICRf male mice. L-Glutamine levels in brain, liver, or L5178Y/AR leukemia cells were the same as controls 4 hours after a single injection of 100 mg/kg of NSC-163501 in BDF_1 mice (2). In contrast, these investigators reported that L-threonine and L-glutamine levels rose markedly in extracts of pancreas in the treated mice. These differences probably depend on the pretreatment amino acid levels, their rate of utilization, and the activities of enzymes inhibited by NSC-163501.

In mice infected with the lactate dehydrogenase-elevating virus, *Acinetobacter* L-glutaminase-L-asparaginase has a plasma half-life of > 12 hours (8). A single dose of the enzyme depletes circulating levels of L-glutamine and L-asparagine for several days (15). The plasma half-life of NSC-163501 is about 45 minutes in mice (17). The duration of inhibition of target enzymes by this drug or the duration of the potentiation of its effect by L-glutamine depletion remains to be determined. Further experiments are planned with intermittent dose schedules of enzyme and this drug.

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Effects of Methionine Sulfoximine Analogs on the Synthesis of Glutamine and Glutathione: Possible Chemotherapeutic Implications

Alton Meister* and Owen W. Griffith [1]

SUMMARY

Methionine sulfoximine inhibits the synthesis of glutamine, and also, by virtue of its inhibition of γ-glutamylcysteine synthetase, the synthesis of glutathione. The studies presented in this paper on methionine sulfoximine and its analogs have potential chemotherapeutic interest because (*a*) there is evidence that depletion of a specific amino acid (eg, asparagine or glutamine) may have beneficial therapeutic effects, and (*b*) certain tumors (eg, those of the liver, skin, and colon) exhibit high levels of γ-glutamyl transpeptidase, the enzyme that catalyzes the degradation of glutathione according to the γ-glutamyl cycle. Some tumors have high levels of glutathione, and both glutathione levels and γ-glutamyl transpeptidase activities are reported to be increased in tumors, as well as after administration of carcinogens. Inhibition of the γ-glutamyl cycle, which has been postulated to play a role in the transport of amino acids, may reduce amino acid transport into certain tumors. Depletion of glutathione might make tumors more susceptible to radiation or chemotherapeutic agents. The γ-glutamyl derivatives of some chemotherapeutic agents might be transported into certain tumors more readily than the free agents. Specific inhibitors of the individual reactions of the γ-glutamyl cycle have been obtained. Substrate analogs have been found that function in some but not all of the reactions. Methionine sulfoximine inhibits glutamine synthetase and γ-glutamylcysteine synthetase irreversibly by forming enzyme-bound methionine sulfoximine phosphate. An understanding of the mechanisms of action of these enzymes and a knowledge of the topology of their active sites have led to the synthesis of two highly selective sulfoximine inhibitors. Thus, α-ethylmethionine sulfoximine was prepared and was found to inhibit glutamine synthetase in vitro and in vivo, but was also found to be essentially inactive toward γ-glutamylcysteine synthetase. Administration of α-ethylmethionine sulfoximine leads to substantial decreases in tissue glutamine levels without affecting glutathione levels appreciably. Studies on the mapping of the active sites of the two synthetases led to the prediction that substitution of bulkier alkyl groups for the *S*-methyl group of methionine sulfoximine would yield a selective inhibitor of γ-glutamylcysteine synthetase. This prediction was fulfilled by the finding that prothionine sulfoximine (*S*-*n*-propyl homocysteine sulfoximine) is an excellent inhibitor of γ-glutamylcysteine synthetase and only a very weak inhibitor of glutamine synthetase. It has thus been possible, by suitable structural modification, to direct an inhibitor to one enzyme in preference to another very similar enzyme, despite the fact that both enzymes act by closely analogous mechanisms involving formation of the same enzyme-bound intermediate. This approach might be usefully applied to other powerful enzyme inhibitors such as azaserine or 6-diazo-5-oxo-L-norleucine, and thus might provide reagents that would inhibit specific enzyme targets.

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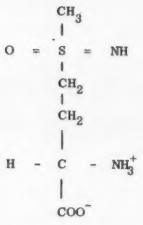


FIGURE 1.—Structure of methionine sulfoximine.

Recent studies in our laboratory have been concerned with the inhibitory effects of methionine sulfoximine (fig 1) and several related compounds on the synthesis of glutamine and glutathione (1–6). These studies may be of interest in connection with cancer chemotherapy for at least two reasons. First, a number of studies (including several reported in this issue) suggest that depletion of a specific amino acid, for example, glutamine or asparagine, may have favorable therapeutic effects. Administration of methionine sulfoximine to animals inhibits glutamine synthetase activity, and is therefore associated with a marked decrease in the concentration of tissue glutamine. (This effect can be associated with severe toxicity as discussed below.) Second, glutathione is not required in the diet, but this tripeptide (γ-glutamyl-cysteinyl-glycine) is present in millimolar concentrations in virtually all living cells. Its function in detoxification, in oxidation-reduction phenomena, in amino acid transport, and as a major source of cysteine for protein synthesis has been extensively examined (5,7–9). It is notable that a number of tumors contain very high concentrations of glutathione and that tumors also exhibit high levels of γ-glutamyl transpeptidase, the enzyme that catalyzes the first step in the breakdown of glutathione according to the γ-glutamyl cycle (fig 2) (10). This cycle, which accounts for the biosynthesis and utilization of glutathione, appears to be one of the systems that mediates the transport of amino acids in mammalian tissues (5). Inhibition of the cycle might reduce amino acid transport into certain tumors. In addition, tumor cells depleted of glutathione might be more susceptible to radiation or to chemotherapeutic agents because glutathione-dependent detoxification pathways would be reduced. It is also possible that the γ-glutamyl derivatives of some chemotherapeutic agents might be transported into certain tumors more readily than the agents themselves. Thus, a chemotherapeutic approach directed to the glutathione system and the γ-glutamyl cycle would seem desirable.

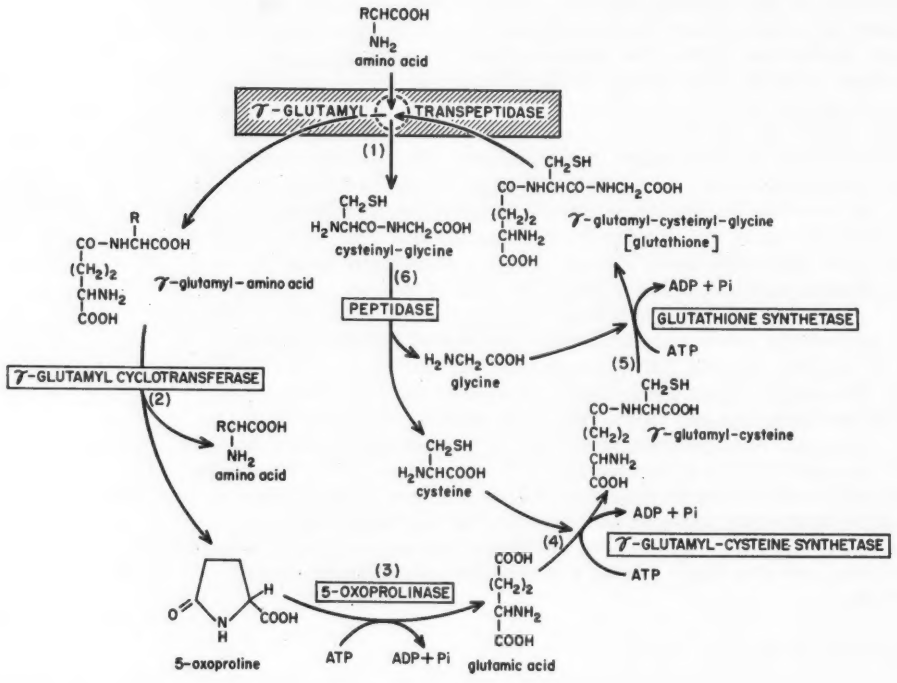


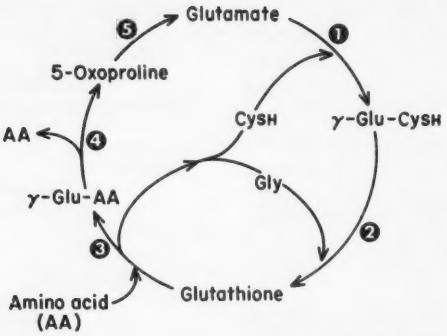
FIGURE 2.—γ-Glutamyl cycle (*from* ref 10).

TABLE 1.—Glutathione, γ-glutamyl transpeptidase, and cancer

Treatment and/or tumor	Concentration of—		Ref Nos.
	Glutathione	γ-Glutamyl transpeptidase	
3′-Methyl-4-dimethylaminoazobenzene	Increased		11
Correlation with azo dye carcinogenicity	Increased		12
Transplantable rat hepatomas		Increased	13
2-Acetylaminofluorene, ethionine, others		Increased	14, 15
Ligandin binds carcinogens	*S*-Transferase		9
Carcinogens, Morris hepatomas	Increased		16
Mouse skin carcinogens	Increased		17
Human skin tumors	Increased		17
1,2-Dimethylhydrazine-induced colon tumors		Increased	18
Hepatoma tissue culture cells		Increased	19
2-Acetylaminofluorene, diethylnitrosamine-premalignant cells		Increased	20

There are now a number of reports indicating a connection between glutathione metabolism and carcinogenesis (table 1). For example, the concentration of glutathione in male rat liver increases after administration of 3′-methyl-4-dimethylaminoazobenzene (11), and a good correlation has been found between the relative carcinogenic activities of azo dyes and the ability of these compounds to increase the concentration of liver glutathione (12). The simultaneous occurrence of dye binding and increased glutathione concentration seems to be essential for azo dye hepatocarcinogenesis. Certain chemically induced transplantable rat hepatomas have remarkably high levels of γ-glutamyl transpeptidase (13). The γ-glutamyl transpeptidase activity of liver increases after administration of 3′-methyl-4-dimethylaminoazobenzene, 2-acetylaminofluorene, thioacetamide, ethionine, and dimethylnitrosamine (14,15). Administration of hepatocarcinogens to rats leads to increased levels of liver glutathione and increased γ-glutamyl transpeptidase activity (16). Application of certain polycyclic hydrocarbons to mouse skin leads to a significant increase in glutathione content (17). The glutathione content of human skin carcinomas is reported to be substantially increased (17). It has been concluded that "the level of tissue glutathione is an important biochemical parameter that correlates positively with tumor growth rate in human skin carcinoma" (17). Other studies have shown that colon adenocarcinomas induced by 1,2-dimethylhydrazine have a much higher γ-glutamyl transpeptidase activity than do the normal tissues of origin (18). 1,2-Dimethylhydrazine-induced colonic tumors resemble human colonic adenocarcinomas, and it has been suggested that assays of the transpeptidase activity of human colon mucosa might be of potential value in diagnosing neoplastic change. Studies on the γ-glutamyl transpeptidase activity of normal, regenerating, and malignant hepatocytes have shown that the isolated normal hepatocytes exhibit relatively low transpeptidase activity, but that hepatoma tissue culture cells have considerably increased activity. The rapidly growing Morris hepatoma 7777 also exhibits high γ-glutamyl transpeptidase activity (19). Studies carried out by enzymatic and histochemical staining procedures on different cell populations during induction of liver cancer with 2-acetylaminofluorene and diethylnitrosamine showed a very marked increase in enzyme activity in the tumor nodules compared to controls. It is notable that the activity increases in foci of very early putative preneoplastic hepatocytes induced by a single dose of carcinogen. It has been concluded that increased γ-glutamyl transpeptidase activity is a marker for very early, as well as late, putative liver preneoplastic and neoplastic cells (20). It is also of interest that ligandin, a protein that exhibits glutathione *S*-transferase activity, binds certain carcinogens effectively (9).

The studies presented here on methionine sulfoximine analogs developed from two closely associated lines of research in our laboratory. In one approach, we attempted to achieve specific inhibitors of the individual reactions of the γ-glutamyl cycle, and also substrate analogs that would function in some, but not all, of the individual reactions (21). We anticipated that this might be difficult to accomplish because most of the reactions of the cycle involve the γ-carboxyl group of glutamate and, therefore, a given substrate analog might interact with more than a single enzyme. On the other hand, the individual reaction pathways are different and must therefore be associated with differences in the enzyme-bound conformations of the glutamate carbon chain at the active sites of several enzymes. As summarized in figure 3, we found that suitable modification of the glutamyl moiety of the substrates does yield effective and specific inhibitors or unmetabolizable analogs for each of the steps of the cycle. For example, *N*-methylglutamate and *N*-methyl-γ-glutamyl α-aminobutyrate are substrates of the synthetases and thus lead to formation of an *N*-methylglutathione analog, which is not a substrate for γ-glutamyl transpeptidase. Similarly, β-glutamate is a substrate for γ-glutamylcysteine synthetase and β-glutamyl-α-amino-



Substrate Analogs	ENZYMES ❶ γ-Glu-CysH Synthetase	❷ GSH Synthetase	❸ γ-Glu Trans-peptidase	❹ γ-Glu Cyclo-transferase	❺ 5-Oxoprolinase
D-Glu	0,↓	+	+	0,↓	0
α-Methyl-Glu	(+)	+	+	0	0
N-Methyl-Glu	+	+	0	0	0
β-Glu	+	+	0	0,↓	
β-Methyl-Glu	(+),↓	+,↓	0	+	0,↓
γ-Methyl-Glu	0,↓	+	0	0	(+),↓

↓ = >50% inhibition; + = >10% as active as L-glu;
(+) = 1–10% as active as L-glu; 0 = <1% as active as L-glu

FIGURE 3.—Interaction of glutamate and glutamyl analogs with enzymes of the γ-glutamyl cycle. CysH = cysteine; GSH = glutathione (*from* ref 21).

butyrate is a substrate for glutathione synthetase, but the resulting glutathione analog is not a substrate for the transpeptidase, nor is β-glutamyl-α-aminobutyrate a substrate for γ-glutamyl cyclotransferase. β-Glutamyl-α-aminobutyrate is in fact an excellent inhibitor of γ-glutamyl cyclotransferase (as is D-γ-glutamyl-L-α-aminobutyrate). Figure 3 describes other effects of various substrate analogs on five enzymes of the γ-glutamyl cycle.

Another experimental approach has developed from our interest in the mechanism of action of glutamine synthetase (22). In the course of this work, we examined the mechanism by which methionine sulfoximine irreversibly inhibits the enzyme. Studies on methionine sulfoximine began about 30 years ago at a time when nitrogen trichloride (agene) was used as a bleaching agent for flour. It was found that dogs fed such agenized flour developed convulsions and that the convulsant was methionine sulfoximine produced by the action of nitrogen trichloride on protein-bound methionine residues. It is now known that methionine sulfoximine produces convulsions in many animal species. As discussed further below, the available data led to the conclusion that methionine sulfoximine-induced convulsions are closely associated with the inhibition of glutamine synthetase.

Studies on the inhibition of glutamine synthetase by methionine sulfoximine showed that this amino acid derivative inhibits the enzyme by interacting with adenosine triphosphate to form enzyme-bound methionine sulfoximine phosphate (1–3,5). Methionine sulfoximine phosphate remains firmly attached to the enzyme, thus inhibiting it irreversibly. It is of interest that only one of the four isomers of methionine sulfoximine (L-methionine-*S*-sulfoximine) inhibits glutamine synthetase (3) and that only the same isomer induces convulsions in mice (23). Studies on the mapping of the active site of glutamine synthetase have demonstrated the close similarity between the calculated structure of the tetrahedral intermediate or transition state formed in the attack of ammonia on enzyme-bound γ-glutamyl phosphate and the structure of methionine sulfoximine phosphate (22,24,25). Thus, the arrangement of atoms about the sulfur atom of methionine sulfoximine is tetrahedral and closely mimics the geometry of the tetrahedral intermediate presumably formed in the normal catalytic reaction. These studies have thus provided substantial support for the intermediate formation of an acyl phosphate, ie, γ-glutamyl phosphate, in the normal reaction.

Subsequent studies in our laboratory on γ-glutamylcysteine synthetase provided evidence that the mechanism of this reaction also involves intermediate formation of

enzyme-bound γ-glutamyl phosphate (26); for this reason, the effect of methionine sulfoximine on the γ-glutamylcysteine synthetase reaction was examined. Methionine sulfoximine was found to be an effective inhibitor of γ-glutamylcysteine synthetase. Methionine sulfoximine is converted to methionine sulfoximine phosphate on this enzyme (4), as was previously found with glutamine synthetase. Furthermore, of the four stereoisomers of methionine sulfoximine, only L-methionine-*S*-sulfoximine was inhibitory. The mechanism of inhibition of γ-glutamylcysteine synthetase by L-methionine-*S*-sulfoximine is thus similar to that by which this compound inhibits glutamine synthetase.

Since administration of methionine sulfoximine inhibits the synthesis of both glutamine and glutathione, the convulsant activity of this compound might be due to an effect on glutathione synthesis (or the γ-glutamyl cycle), or to an effect on glutamine synthetase. As expected, the administration of methionine sulfoximine leads to substantial decreases in the glutamine concentration in the brain, liver, and kidney, as well as to comparable decreases in the concentration of glutathione in the liver and kidney. Although methionine sulfoximine does not affect the overall concentration of brain glutathione, it cannot be overlooked that glutathione present in a quantitatively small pool might be severely depleted without significantly affecting the average concentration of glutathione in the brain.

The findings on selective inhibition of the enzymes of the γ-glutamyl cycle by various substrate analogs [fig 3; (21)] suggested the possibility of designing specific inhibitors of glutamine synthetase and γ-glutamylcysteine synthetase. Thus, we thought that derivatives of methionine sulfoximine might be prepared which would exhibit one-enzyme specificity. We have indeed succeeded in obtaining a specific inhibitor of glutamine synthetase which does not interfere significantly with glutathione synthesis (6), and have also achieved the reciprocal goal of preparing a sulfoximine derivative which inhibits glutathione synthesis without affecting the synthesis of glutamine substantially (27,28). In the course of this work, we prepared a large number of methionine sulfoximine analogs, including α-methylmethionine sulfoximine and α-ethylmethionine sulfoximine. The effects of the α-alkyl derivatives on glutamine synthetase and on γ-glutamylcysteine synthetase are shown in figure 4 (6). α-Methylmethionine sulfoximine was about as effective, or perhaps slightly more effective (assuming that only one diastereoisomer is active), as methionine sulfoximine in inhibiting glutamine synthetase, whereas α-ethylmethionine sulfoximine was less active than methionine sulfoximine in inhibiting this enzyme. However, α-ethylmethionine sulfoximine did not inhibit γ-glutamylcysteine synthetase significantly (fig 4B, curve 4). The glutamine levels of the liver and kidney were substantially decreased after administration of methionine sulfoximine, α-methylmethionine sulfoximine, and α-ethylmethionine sulfoximine. Similar results were found in brain except that α-ethylmethionine sulfoximine produced only a slight decrease in brain glutamine. The glutathione levels of the liver and kidney (but not those of the brain) were markedly decreased after administration of methionine sulfoximine and α-methylmethionine sulfoximine. It is notable that α-ethylmethionine sulfoximine did not affect the glutathione levels of the liver and kidney significantly.

The findings thus indicate that modification of the methionine sulfoximine molecule by introduction of an α-ethyl group alters the specificity of inhibition such that this agent inhibits only one of the synthetases, ie, glutamine synthetase. Since α-ethylmethionine sulfoximine produces convulsions, the data support the conclusion that the convulsant effect of methionine sulfoximine itself is closely related to its inhibition of glutamine synthetase rather than of γ-glutamylcysteine synthetase.

Earlier studies (29) showed that methionine sulfoximine is converted in vivo into a number of *S*-containing products. Studies with L-amino acid oxidase, glutamine transaminase, and cystathionase showed that methionine sulfoximine can be converted into the corresponding α-keto and α-imino acids which undergo γ-elimination to yield a number of compounds including methane sulfinimide, methane sulfonamide, methane sulfinic acid, methane sulfonic acid, 2-imino-3-butenoic acid, and vinyl glyoxylate (30). These findings are consistent with the possibility that convulsions are produced by a metabolite (or metabolites) of methionine sulfoximine. However, the α-alkyl analogs of methionine sulfoximine cannot undergo transamination and, like other α-substituted amino acids, are probably not metabolized to a significant extent in vivo. Thus, the convulsant activity of the α-alkyl derivatives of methionine sulfoximine (and by implication, also of methionine sulfoximine) is most probably due to the sulfoximine molecules themselves rather than to their metabolites. Furthermore, both stereoisomers of L-methionine-*SR*-sulfoximine are substrates for glutamine transaminase and amino acid oxidase (30) while, as noted, only L-methionine-*S*-sulfoximine causes convulsions.

In an attempt to obtain a derivative of methionine sulfoximine that would affect glutathione synthesis without perturbing the synthesis of glutamine, we were guided by previous studies on the mapping of the active site of glutamine synthetase in which it was shown that the *S*-methyl group of methionine sulfoximine attaches to the enzyme site that binds ammonia in the normal catalytic reaction (24). We therefore thought that substitution of a bulkier group in place of the methyl group of methionine sulfoximine might interfere with attachment of the inhibitor molecule to glutamine synthetase. Furthermore, the comparable region of the active site of γ-glutamylcysteine synthetase would be expected to bind a somewhat

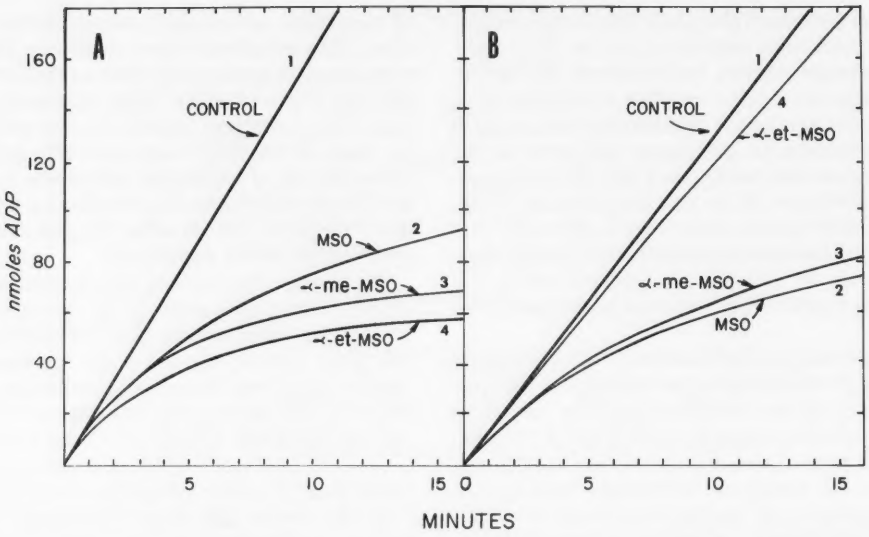


FIGURE 4.—Time course of reactions catalyzed by glutamine synthetase (A) and γ-glutamylcysteine synthetase (B) in the presence of substrates and inhibitors. *A*. The reaction mixtures contained (final volume, 1.0 ml) 50 mM imidazole·HCl buffer (pH 7.25), 10 mM adenosine triphosphate, 5 mM phosphoenolpyruvate, 20 mM $MgCl_2$, 5 mM sodium-L-glutamate, 40 mM $(NH_4)_2SO_4$, 0.2 mM nicotinamide adenine dinucleotide (reduced form), 4 units of pyruvate kinase, 5.5 units of lactate dehydrogenase, and 0.18 unit of glutamine synthetase. Curve 1 gives the time course of the reaction without inhibitor. In the other reactions, inhibitor was present as follows: curve 2, 0.2 mM L-methionine-*SR*-sulfoximine (MSO); curve 3, 0.4 mM α-methyl-DL-methionine-*SR*-sulfoximine (α-me-MSO); curve 4, 10 mM α-ethyl-DL-methionine-*SR*-sulfoximine (α-et-MSO). *B*. The reaction mixtures contained (final volume, 1.0 ml) 150 mM Tris·HCl buffer (pH 8.2), 75 mM KCl, 10 mM adenosine triphosphate, 5 mM phosphoenolpyruvate, 25 mM $MgCl_2$, 5 mM sodium-L-glutamate, 10 mM L-α-aminobutyrate, 0.2 mM nicotinamide adenine dinucleotide (reduced form), 0.2 mM EDTA, 4 units of pyruvate kinase, 5.5 units of lactate dehydrogenase, and 0.77 unit of γ-glutamylcysteine synthetase. Curve 1 gives the time course of the reaction without inhibitor. In the other reactions, inhibitor was present as follows: curve 2, 0.2 mM L-methionine-*SR*-sulfoximine; curve 3, 0.4 mM α-methyl-DL-methionine-*SR*-sulfoximine; curve 4, 10 mM α-ethyl-DL-methionine-*SR*-sulfoximine (*from* ref 6).

bulkier moiety than a methyl group since the acceptor amino acid (cysteine or α-aminobutyrate) is considerably larger than the ammonia or the methyl group. This line of reasoning was supported by the finding that ethionine sulfoximine exhibits relatively more inhibition toward γ-glutamylcysteine synthetase than toward glutamine synthetase. Ethionine proved to be a weak convulsant agent. We therefore prepared *S-n*-propyl homocysteine sulfoximine and found that this compound is an excellent inhibitor of γ-glutamylcysteine synthetase and only a rather weak inhibitor of glutamine synthetase. The *S-n*-propyl derivative produces convulsions only when given at doses 100 times higher than the dose of methionine sulfoximine which produces convulsions. It is of interest that the *S-n*-propyl derivative is a more effective inhibitor of γ-glutamylcysteine synthetase than are ethionine sulfoximine and methionine sulfoximine. These investigations, which will be described in detail elsewhere (28), have thus provided us with a potentially valuable inhibitor for further work on the effects of glutathione depletion under conditions in which glutamine levels remain relatively unaltered.

These studies, which have led us to effective and specific one-enzyme inhibitors that are active in vivo, indicate that it is possible by suitable structural modification to direct an inhibitor to one enzyme in preference to another very similar enzyme, even though both enzymes act by closely analogous mechanisms and involve intermediate formation of the same enzyme-bound acyl phosphate intermediate. This approach might possibly be applied to other powerful enzyme inhibitors such as azaserine and 6-diazo-5-oxo-L-norleucine, which inhibit many glutamine amidotransferases (31) as well as γ-glutamyl transpeptidase (32). For example, they inhibit such enzymes as glutamine-dependent carbamyl phosphate synthetase, formylglycinamidine ribonucleotide synthetase, 5-phosphoribosyl pyrophosphate amidotransferase, cytidine triphosphate synthetase, asparagine synthetase, guanosine monophosphate synthetase, and nicotinamide adenine dinucleotide synthetase. Although azaserine and 6-diazo-5-oxo-L-norleucine act as potent irreversible inhibitors, it is clear that they are nonspecific in their metabolic effects. We suggest that appropriate structural modification of these compounds might yield drugs that would hit single-enzyme targets and thus hopefully be more useful in chemotherapy.

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Molecular Engineering of the L-Tryptophan-Depleting Enzyme Indolyl-3-alkane α-Hydroxylase[1]

Gottfried Schmer[2,*] and Joseph Roberts[3,4]

SUMMARY

Polymaleic acid and polyacrylic acid polymers have been covalently linked to indolyl-3-alkane α-hydroxylase with a shift of the pH optimum from 3.5 to between 5.0 and 5.5. In addition, the specific activity of the modified enzyme was increased approximately threefold at pH 7.0. The K_m values of the polymer-bound enzyme preparations are essentially the same as that of the native enzyme. The plasma half-life was shortened from 6–7 hours with the native enzyme to 2–4 hours with the modified enzymes. The degree of L-tryptophan depletion in the circulation of mice, however, was the same during a 24-hour period after injection of native or modified enzymes.

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The molecular engineering of amino acid-depleting enzymes to abolish undesirable side effects has gained increasing importance in the last few years. The dramatic increase of biologic half-life in mice of *Acinetobacter* L-glutaminase-L-asparaginase achieved by succinylation and glycosylation (1), the increase of tissue specificity of amino acid-depleting enzymes by taking advantage of specific membrane receptor interaction with a carrier protein attached to the enzyme (2), and the recent demonstration of a dramatic decrease in the immunogenicity of proteins (3,4) by the covalent attachment of polyethyleneglycol are testimony to the successful manipulation of amino acid-depleting enzymes by chemical modifications.

A novel L-tryptophan-depleting enzyme, indolyl-3-alkane α-hydroxylase, was recently isolated and characterized (5,6). This novel enzyme showed antitumor activity against certain mouse sarcomas and carcinomas (7). A major disadvantage of this enzyme was that its pH optimum fell at 3.5, with only about a tenth of the maximal rate at neutral pH.

This paper describes the shift of the optimal enzyme rate of about 2 pH units towards neutrality by covalently linking polyacrylic acid to the enzyme. When soluble polymaleic acid was bound to the enzyme, a flattening of the pH-activity profile occurred towards neutral pH. In both cases the chemical modification resulted in about a threefold increase in the specific activity at a pH of 7.0.

MATERIALS AND METHODS

Indolyl-3-alkane α-hydroxylase was isolated as described previously (5). Enzyme activity was determined by the reagent C color assay (5). Protein was measured by the method of Lowry et al (8).

Polyacrylic acid (molecular weight, 250,000), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide, and *N*-hydroxysuccinimide were purchased from Aldrich Chemical Co. Polymaleic acid anhydride (molecular weight, 98,000) was obtained from Polysciences, Inc. Bio-Gel A5m (100–200 mesh) was used to separate the enzyme-polymer compound from free polymer. The dimethylformamide was spectroscopy grade, and all other chemicals used were reagent grade.

Synthesis of Polyacrylic Acid-Indolyl-3-alkane α-Hydroxylase

Synthesis of polyacrylic acid ester with N-hydroxysuccinimide.—Four g of polyacrylic acid, 2 g of carbodiimide, and 2 g of *N*-hydroxysuccinimide were dissolved

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in 100 ml of water-free dimethylformamide. The reaction to form the intermediate ester was allowed to take place for at least 100 hours at room temperature in well-stoppered glass bottles. The polyacrylic *N*-hydroxysuccinimide ester was stored at 4°C, tightly sealed against moisture with parafilm prior to use.

Synthesis of polyacrylic acid-indolyl-3-alkane α-hydroxylase.—Twenty mg of purified enzyme (200–300 units) in 10 ml of 0.025 M sodium acetate, pH 5.5, was rapidly mixed with 1 ml of polyacrylic acid ester as described above (representing at least a twofold weight excess or molar excess of polyacrylic acid over the enzyme). The pH was maintained at 5.5 by rapid dropwise addition of 1 N NaOH to the reaction mixture. After a rapid initial hydrolysis of the ester with a drop of pH in the first few minutes, there was only a minor drop thereafter. Hydrolysis or binding to the enzyme, respectively, seemed to be complete after 60 minutes. The reaction was carried out in an appropriate glass beaker in an ice water bath. When no further drop in the pH was observed, the mixture of hydrolyzed-free polymer and enzyme-bound polymer was dialyzed against 1000 volumes of 0.025 M sodium acetate (pH 5.5) at 4°C overnight. The polymer plus polymer-enzyme mixture could then be deep frozen at –80°C without loss of activity. Five-ml samples containing free and enzyme-bound polymer were passed over Bio-Gel columns (4 × 120 cm) at 4°C to separate free from enzyme-bound polymer. A small sample of the polymer ester hydrolyzed in the presence of L-[^{14}C]-leucine was added (~ 0.05 microcurie) as a marker to indicate the degree of separation from the polymer-enzyme compound.

Synthesis of polymaleic acid-indolyl-3-alkane α-hydroxylase.—Twenty mg of purified enzyme in 10 ml of 0.025 M sodium acetate, pH 5.5, was rapidly mixed with 200 mg of polymaleic acid anhydride dissolved in 2 ml of water-free dimethylformamide. Again, the pH was rapidly re-adjusted to 5.5 with 1 N NaOH after the rapid initial hydrolysis of the polymer. Further hydrolysis was slow and seemed complete after 60 minutes. Throughout the reaction the pH was kept constant at 5.5 with both polymers described. Dialysis or separation of enzyme-bound and free polymer was carried out exactly as described for polyacrylic acid. Again, an L-[^{14}C]-leucine marker was used to ascertain complete separation of free and bound polymer. The mixture could again be frozen at –80°C without loss of activity prior to column separation. After gel filtration, the diluted polymer-enzyme fraction was concentrated over Amicon Diaflo ultrafilter membranes (PM30) to about the original enzyme concentration (20–30 units/ml) and immediately deep frozen at –80°C.

Estimation of K_m of Nonmodified and Modified Indolyl-3-alkane α-Hydroxylase

The K_m values of nonmodified enzyme and enzyme bound to the acidic polymers were determined at pH 5.5 (0.1 M sodium acetate) by trapping and counting [^{14}C]-O_2 released from L-[1-^{14}C]-tryptophan as previously described (5).

Determination of Plasma Half-Life of Nonmodified Indolyl-3-alkane α-Hydroxylase and the Enzyme Coupled to Acidic Polymers

One hundred and fifty units per kg of body weight of nonmodified or modified enzyme was injected through the tail veins of non-tumor-bearing CD-1 female mice. Blood for determination of plasma indolyl-3-alkane α-hydroxylase activity was obtained by the orbital bleeding technique. The enzyme activity was assayed by measuring [^{14}C]-O_2 evolution from L-[1-^{14}C]-tryptophan (5).

RESULTS

Figure 1 shows the enzyme-activity profile as a function of pH of nonmodified enzyme (average of eight experiments), enzyme bound to soluble polymaleic acid polymers (11 experiments), and enzyme bound to soluble polyacrylic acid polymers (13 experiments). The three curves reflect about the same protein concentration of highly purified enzyme. It is evident that there is about a threefold increase in specific activity of the enzyme coupled to acidic polymers at pH 7.0, which has an obvious significance in biologic experiments. Table 1 illustrates that there is no significant change in the K_m values of the enzymes bound to the acidic polymers as compared to nonmodified enzymes. In spite of the appreciable change in physiochemical characteristics, the high substrate affinity seems to show little or no change.

Plasma half-life of the modified enzymes is appreciably shortened (table 2), especially in the case of polymaleic acid-indolyl-3-alkane α-hydroxylase (2 hours). Polyacrylic acid-indolyl-3-alkane α-hydroxylase shows a plasma half-life of 4 hours as compared to 6–7 hours for nonmodified enzyme.

Table 3 summarizes the degree of L-tryptophan depletion in plasma after the administration of nonmodified enzyme, and enzyme bound to acidic polymers. Although the enzyme polymers show a shorter half-life than the native enzyme, there is no appreciable difference in plasma L-tryptophan depletion through the duration of the experiment (18–25 hours).

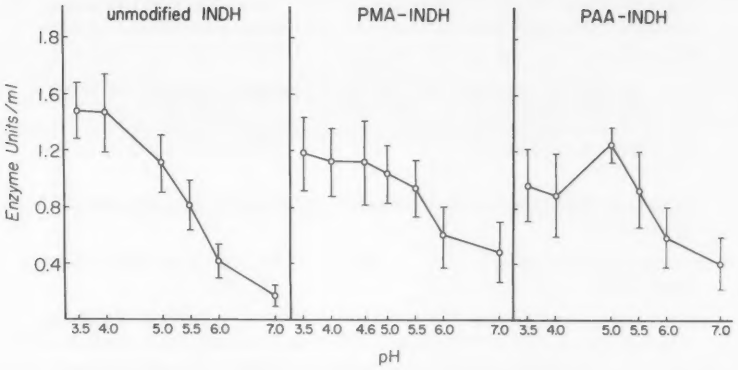

FIGURE 1.—Enzyme activity of nonmodified indolyl-3-alkane α-hydroxylase (INDH, average of 8 experiments), polymaleic acid-indolyl-3-alkane α-hydroxylase (PMA-INDH, average of 11 experiments), and polyacrylic acid-indolyl-3-alkane α-hydroxylase (PAA-INDH, average of 13 experiments) as a function of pH. Enzyme activity was measured by the reagent C color assay (5).

TABLE 1.—K_m values of nonmodified indolyl-3-alkane α-hydroxylase and of enzyme bound to soluble polyacrylic acid and polymaleic acid polymers*

Enzyme preparation	K_m
Nonmodified indolyl-3-alkane α-hydroxylase	2.4×10^{-6} M
Polyacrylic acid-indolyl-3-alkane α-hydroxylase	7.0×10^{-6} M
Polymaleic acid-indolyl-3-alkane α-hydroxylase	3.4×10^{-6} M

*The K_m for L-tryptophan was determined at pH 5.5 (0.1 M sodium acetate) by trapping and counting $[^{14}C]$-O_2 released from L-[1-^{14}C]-tryptophan. The specific activity was 1 microcurie/μmol. The amount of enzyme activity was adjusted so that < 25% of the substrate was converted to product. The reaction mixtures (0.5 ml) were incubated at 37°C.

TABLE 2.—Plasma half-lives of nonmodified indolyl-3-alkane α-hydroxylase and of enzyme bound to soluble polyacrylic acid and polymaleic acid polymers*

Enzyme preparation	Half-life (hrs)
Nonmodified indolyl-3-alkane α-hydroxylase	6
Polyacrylic acid-indolyl-3-alkane α-hydroxylase	4
Polymaleic acid-indolyl-3-alkane α-hydroxylase	2

*Nonmodified enzyme (150 units/kg of body weight), enzyme coupled to polyacrylic acid (100 units/kg of body weight), and enzyme coupled to polymaleic acid (150 units/kg of body weight) were injected through the tail veins of female CD-1 mice. Blood for the determination of half-lives was obtained by the orbital bleeding technique after 1, 3, 5, and 6 hrs.

DISCUSSION

The present data indicate that the binding of acidic, soluble polymers, such as polymaleic acid and polyacrylic acid, cause an appreciable shift of optimal enzyme rate from pH 3.5 to pH 5.0–5.5 with an increase in specific activity at pH 7.0. It may be postulated that the formation of a "microaura" of acidic groups around the catalytic site creates a local pH change with more favorable conditions for substrate-product conversion, while the enzyme otherwise might be in a neutral milieu. It is evident that this local pH shift near the catalytic site is greatly influenced by the density and proximity of the acidic groups. Only two molecular sizes of acidic polymers have been used (polyacrylic acid, molecular weight, 250,000; and polymaleic acid, molecular weight, 98,000). Binding of acidic polymers of lower molecular weight, perhaps achieving a closer "fit" to the catalytic site, and binding at different pHs, notably neutral and alkaline, might further improve the shift of maximal enzyme rate toward neutrality.

The fact that polymaleic acid-indolyl-3-alkane α-hydroxylase is able to markedly decrease the plasma L-tryptophan level for 24 hours in spite of the relatively short plasma half-life of 2 hours, suggests that the modified enzyme is trapped somewhere in the body and continues to exert an influence on the L-tryptophan levels in plasma.

TABLE 3.—Plasma L-tryptophan levels after injection of nonmodified indolyl-3-alkane α-hydroxylase and enzyme bound to soluble polyacrylic acid and polymaleic acid polymers

Enzyme preparation	Dose (units/kg of body weight)	Plasma L-tryptophan levels in nmols/ml of plasma*
Nonmodified indolyl-3-alkane α-hydroxylase	400	100 (0), 0.66 (1), 0.66 (7), 5.3 (19)
Nonmodified indolyl-3-alkane α-hydroxylase	500	74 (0), 2.4 (1), 3.2 (7), 21 (24)
Nonmodified indolyl-3-alkane α-hydroxylase	600	122.3 (0), 2.5 (1), 3.7 (6), 6.0 (16), 7.7 (25)
Polymaleic acid-indolyl-3-alkane α-hydroxylase	150	120 (0), 3.5 (1), 4.1 (5), 4.9 (6½), 13.3 (24)
Polyacrylic acid-indolyl-3-alkane α-hydroxylase	100	120 (0), 4.8 (2), 5.8 (5), 7.1 (6½), 12.7 (22)

*Values in parentheses represent hrs after administration. All enzyme preparations were injected through the tail veins of female CD-1 mice. Bleeding was done by orbital puncture. Plasma L-tryptophan levels were determined by a modified fluorometric technique (10).

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Treatment of L5178Y Tumor-Bearing BDF_1 Mice With a Nonimmunogenic L-Glutaminase-L-Asparaginase[1]

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SUMMARY

An L-glutaminase-L-asparaginase from *Achromobacter* has been rendered nonimmunogenic by the covalent attachment of polyethylene glycol (PEG) to nonessential amine groups of the enzyme. PEG-L-glutaminase-L-asparaginase exhibits a greatly enhanced half-life in the bloodstream compared to the unmodified enzyme in normal mice, and is effective in prolonging the survival of BDF_1 mice inoculated ip with L5178Y cells. PEG-L-glutaminase-L-asparaginase appears rapidly in the blood following ip injection.

[Cancer Treat Rep 63:1127–1132, 1979]

A major limitation to the use of L-asparaginase or L-glutaminase-L-asparaginase for the treatment of leukemias has been the immunogenicity of the enzymes, which limits their effective use to one or a few injections (1–3). L-Asparaginase or L-glutaminase-L-asparaginase ordinarily is of little or no value when given as a second series of injections for the treatment of relapses (4). We find that the covalent attachment of strands of polyethylene glycol (PEG) to several enzymes (catalase [5], uricase from two sources,[3] L-arginase,[4] trypsin,[5] superoxide dismutase [6], and adenosine deaminase [7]) renders these enzymes nonimmunogenic as determined by criteria such as nonproduction of antibodies against the native or modified enzymes or by unaltered blood circulating lives after repetitive long-term iv injections. In every instance, the circulating half-life in the bloodstream is greatly extended over that of the native enzyme, even on first injection.

This paper reports the preparation of a nonimmunogenic PEG-L-glutaminase-L-asparaginase, and the initial evaluation of its therapeutic effectiveness in treating BDF_1 mice infected with the L5178Y tumor.

MATERIAL AND METHODS

BDF_1 mice were purchased from Jackson Laboratories, Bar Harbor, Me, or from ARS/Sprague-Dawley, Chagrin Falls, Ohio. L5178Y cells were obtained as frozen suspensions from Dr. J. Roberts (Sloan-Kettering Institute for Cancer Research, Rye, NY). The cells were carried in BDF_1 mice in the ascitic form by ip inoculation of 10^6 cells/mouse. After 10 days of growth, cells were reinoculated into a second set of mice. Test mice were infected with cells derived from the second set of mice.

L-Glutaminase-L-asparaginase from *Achromobacter* was kindly supplied by Dr. John Douros, Natural Products Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. A later supply was obtained from the Medical Research Establishment, Porton Down, Salisbury, England. This enzyme has shown antileukemic effects in man (8). Antiserum to the L-glutaminase-L-asparaginase was prepared in New Zealand white female rabbits as described previously for catalase (5). PEG of 5000 daltons was activated for covalent attachment to the enzyme as described by Abuchowski et al (9).

To prepare nonimmunogenic PEG-L-glutaminase-L-asparaginase, 8000 IU of enzyme (150 IU/mg) was dissolved in 8 ml of 0.1 M borate buffer, pH 9.25. Activated PEG (950 mg) was added with stirring, and the solution was left for 2 hours at 18°C. The solution was then dialyzed

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against phosphate-buffered saline, pH 7.3, in the Amicon thin-channel device equipped with the PM-30 membrane, sterilized by passage through a 0.22-μ Millipore filter, and stored at 4°C. The L-asparaginase activity (assayed by the procedure of Jayaram et al [10]) was 12% of the unmodified enzyme. PEG was coupled to 71% of the amino groups, as determined by the method of Habeeb (11).

L-Asparaginase activity is measured by a modification of the coupled enzyme system of Jayaram et al (10). The reaction mixture contains 20 ml of glycerol, 11 mg of α-ketoglutaric acid, 11 mg of NADH, 116 units of L-malate dehydrogenase, 180 units of L-glutamic oxaloacetic transaminase, and 1 mmol of L-asparagine, made up to 100 ml with 0.1 M Tris-HCl, pH 8.35. For assay, 0.9 ml of the reaction mixture is added to a cuvette (1-cm light path; 1-ml capacity), followed by the addition of 0.1 ml of enzyme (0.0004–0.004 unit [10]) made up in 0.1 M Tris-HCl, pH 8.35, containing 1 mg/ml of bovine serum albumin. The reaction rate is followed at 340 nm with a Gilford recording spectrophotometer for 8–10 minutes. The reaction rate is linear after an initial lag period of 2–3 minutes. One L-asparaginase unit corresponds to the breakdown of 1 μmol of NADH/minute at 37°C.

Mice were infected with 10^6 or 10^7 cells. On the following day the mice were injected with saline (controls), L-glutaminase-L-asparaginase, or PEG-L-glutaminase-L-asparaginase. The amounts of enzyme, injection schedules, and injection route are given in the figure legends.

For studies of the blood circulating lives in mice, the native and PEG enzymes were injected into the tail vein and circulating blood levels were monitored for up to 48 hours by taking 0.1-ml samples at intervals from the orbital sinus. The blood was centrifuged and 10 μl of plasma was withdrawn for assay (10).

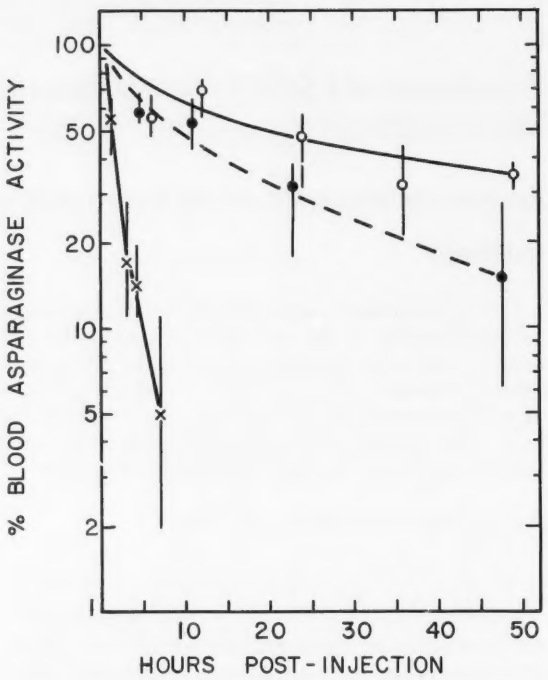

FIGURE 1.—Blood circulating life in mice of L-glutaminase-L-asparaginase and PEG-L-glutaminase-L-asparaginase. Enzymes were injected by tail vein. Blood was collected from the orbital sinus for assay of L-asparaginase activity. Groups of 12 mice were used for each enzyme; each point = the average of 4 mice. Vertical bars = the range of values. PEG-L-glutaminase-L-asparaginase was injected twice weekly at 3- and 4-day intervals. One half of the normal dose was given on the final injection. ×—×—× = circulating life of unmodified L-glutaminase-L-asparaginase after the 1st injection (3 units/mouse). ○—○—○ = PEG-L-glutaminase-L-asparaginase after the 1st injection (3 units/mouse). ●—●—● = PEG-L-glutaminase-L-asparaginase after the 7th injection (1.5 units/mouse).

RESULTS

Clearance Rates and Immunogenicities of L-Glutaminase-L-Asparaginase and Polyethylene Glycol-L-Glutaminase-L-Asparaginase

Figure 1 shows the rate of removal of the native enzyme and the PEG-enzyme from the bloodstream of mice. The circulating half-life of the PEG-enzyme is approximately 24 hours compared to about 2 hours for the native enzyme. Spiers and Wade (8) report a half-life of 80 minutes for the native enzyme in man. After seven iv injections, the PEG-enzyme still shows an extended circulating life. The apparently decreased circulating life seen in figure 1 is a dose effect. Because of a shortage of PEG-enzyme, the amount administered on the final injection was one half that of the first six injections. We commonly see decreased half-lives when the dose is substantially decreased. PEG-L-glutaminase-L-asparaginase did not react with rabbit antibodies against the unmodified enzyme when tested by double immunodiffusion.

Appearance in Blood of L-Glutaminase-L-Asparaginase and Polyethylene Glycol-L-Glutaminase-L-Asparaginase After Ip Injection

PEG-L-glutaminase-L-asparaginase reaches and maintains a higher concentration in blood following ip injection than does the unmodified enzyme (fig 2). Although PEG attachment more than doubles the weight of the molecule, the increased size of the adduct does not appear to limit its entry into the blood. We have also observed this phenomenon with other PEG-enzymes (6,7).

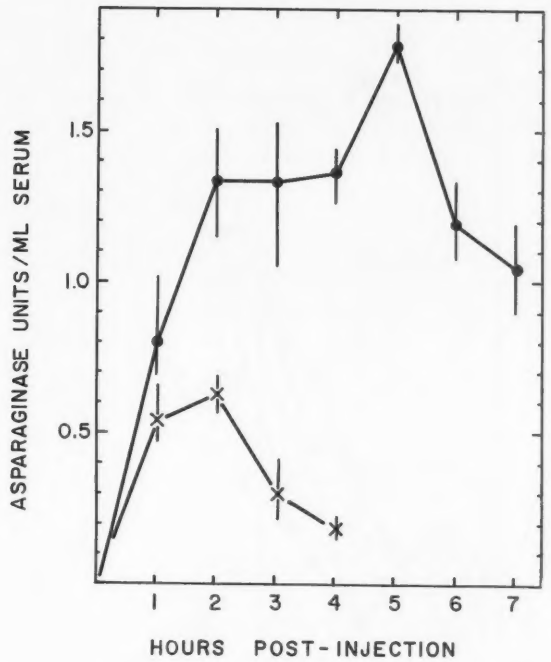

FIGURE 2.—Appearance of unmodified L-glutaminase-L-asparaginase (×—×—×) and PEG-L-glutaminase-L-asparaginase (●—●—●) in the blood following ip injection of 3 units of either enzyme. 12 mice were used for each enzyme; each point = the average of 3 mice. Vertical bars = the range of values.

Therapeutic Effectiveness of Polyethylene Glycol-L-Glutaminase-L-Asparaginase Using Variable Dose Schedules

Initial studies were done on mice that had received a heavy ip inoculation of 10^7 L5178Y cells. Treatment with low levels of PEG-L-glutaminase-L-asparaginase (fig 3) produced a lag period in the weight increase of the animals, but had an insignificant effect on survival time. Survival time could be increased significantly by increasing the dosage level, as shown in figure 4. An attempt was made to maintain a constant weight in each animal; thus, after 10 days on a schedule of 2 units every other day, those mice showing weight increases were given correspondingly larger doses of PEG-L-glutaminase-L-asparaginase (the dosage for each mouse is given on the animal's weight curve in place of the symbol). Increased dosages had the effect of decreasing the animal's weight, often quite drastically. Although the survival time was extended several days, all animals died due to a massive invasion of the L5178Y cells into surrounding organs and tissues.

Figure 5 shows the survival times of mice inoculated with 10^6 L5178Y cells. Again, an attempt was made to adjust the injection schedule so as to stabilize the weight of the animal. Of interest is the sudden decrease in weight of all animals receiving injections of 5 units of PEG-L-glutaminase-L-asparaginase on the 7th day. After the 15th day the animals were treated individually, depending upon their weights. Again, PEG-L-glutaminase-L-asparaginase-treated animals survived significantly longer than the untreated controls. The two necropsied animals exhibited massive infiltration of organs by the tumor cells.

Therapeutic Effectiveness of L-Glutaminase-L-Asparaginase and Polyethylene Glycol-L-Glutaminase-L-Asparaginase Using Fixed Dose Schedules

Survival times of 18–22 days with an inoculum of 10^7 cells occurred in animals receiving 6.5 units ip of PEG-L-glutaminase-L-asparaginase on alternate days for a total of 11 days (six injections). Animals similarly injected with the unmodified enzyme survived 11–15 days. Control animals survived 9–11 days. Doubling the dosage of unmodified L-glutaminase-L-asparaginase to 13 units/injection produced a survival time of 18–23 days, comparable to the survival time of the animals receiving PEG-L-glutaminase-L-asparaginase.

DISCUSSION

The unique property that PEG possesses of extending blood circulating life and eliminating immunogenicity of proteins to which it has been covalently attached is of major potential value for use with therapeutic enzymes. Short circulating lives and adverse immune responses are common problems in enzyme therapy. The increase of L-glutaminase-L-asparaginase half-life in plasma by PEG attachment (24 hours) is superior to the 12–18-hour increase obtained by infection of mice with the LDH virus (12), and eliminates the obvious drawback of a viral infection.

Although the results reported here must be considered preliminary in nature, our results with *Achromobacter* PEG-L-glutaminase-L-asparaginase are sufficiently encouraging that we are expanding our activities to include other host-tumor systems. The modified enzyme was effective in preventing growth of ascitic cells. It did not, however, prevent metastatic growth.

The low catalytic activity of the PEG-L-glutaminase-L-asparaginase in comparison to that of the native enzyme is probably due to excessive modification with PEG. We find that enzymes generally show decreased activities with increasing attachment of PEG. The amount of PEG

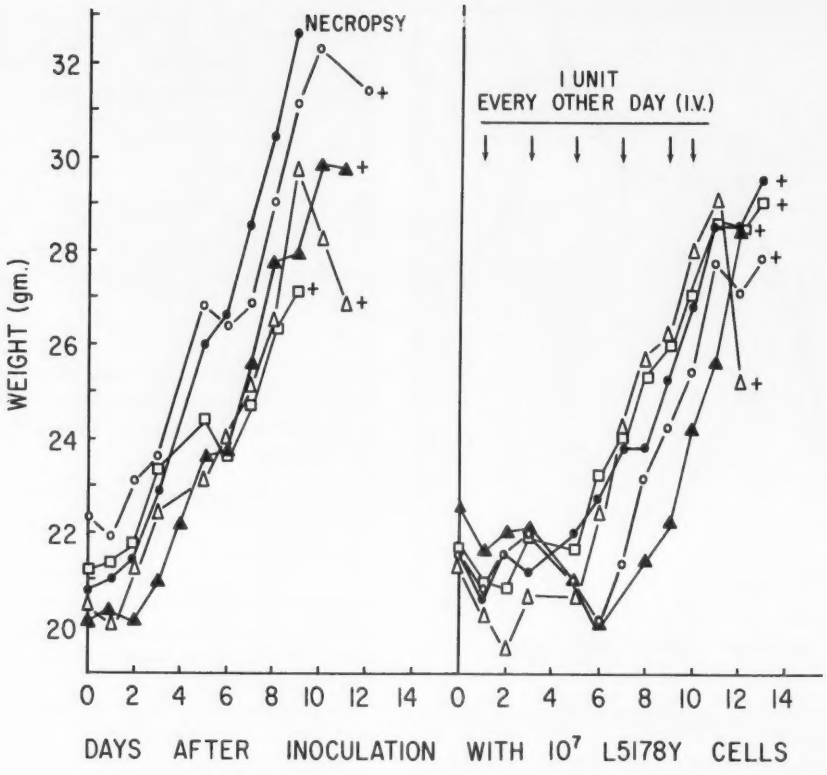

FIGURE 3.—Effect of administering low levels of PEG-L-glutaminase-L-asparaginase on the survival time of BDF_1 mice following ip infection with 10^7 L5178Y cells. 5 mice served as untreated controls. The 5 experimental mice each received 1 unit of PEG-L-glutaminase-L-asparaginase iv on Days 1, 3, 5, 7, 9, and 10 following infection. Left, untreated controls; right, treated animals. A cross = death of the animal. Each symbol = a separate mouse.

attached to this enzyme is in excess of the amount we normally attach, which may account for its low activity. However, it should be noted that we have inactivated some amidohydrolases by PEG attachment. Among these are *Escherichia coli* L-asparaginase EC-2, *Acinetobacter glutaminasificans* L-glutaminase-L-asparaginase (13), and *Vibrio succinogenes* L-asparaginase (14). The *Pseudomonas* 7A L-glutaminase-L-asparaginase described by Roberts (15) retains activity similar to that of the *Achromobacter* enzyme following PEG attachment. Recently, Ashihara et al (16) have used our coupling procedure to attach PEG to an L-asparaginase from *E. coli* A-1-3. Seventy-three of 92 amino groups were modified with PEG-5000, with retention of 7% activity. This preparation did not react against anti-L-asparaginase antiserum. The therapeutic effectiveness of this PEG-L-asparaginase was not tested. Continued searching may turn up an L-asparaginase or L-glutaminase-L-asparaginase that better withstands the PEG modification procedure. Such an enzyme could prove even more useful in cancer therapy.

Elimination of immunologic complications allows the use of PEG-enzymes for extended periods of therapy, alone or in combination with other drugs. The ease with which the PEG-L-glutaminase-L-asparaginase passes from the intraperitoneal cavity into the blood (fig 2) shows that PEG attachment does not inhibit passage of the enzyme through capillary walls. The administration of the PEG-enzyme at one half the dose of the native enzyme produced equal survival times in tumor-bearing animals, indicating that the PEG-enzyme is at least twice as active in vivo as the native enzyme.

Further experiments to quantitate the effectiveness of the modified enzyme using this and other tumor systems are presently underway.

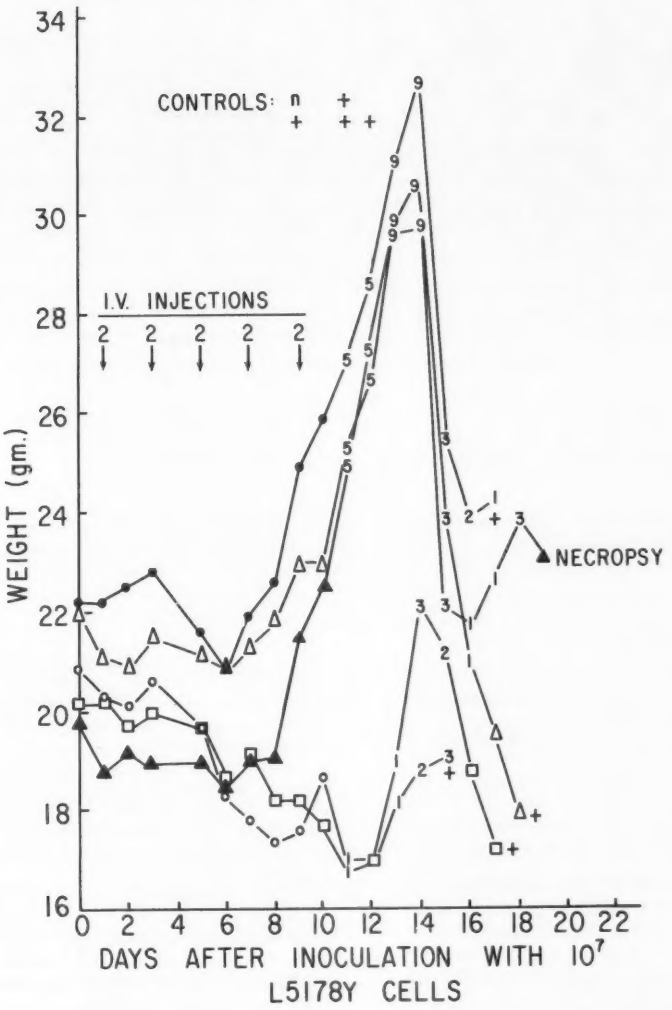

FIGURE 4.—Effect of higher and varied dosages of PEG-L-glutaminase-L-asparaginase on the survival of BDF_1 mice infected ip with 10^7 L5178Y tumor cells. 2 units of enzyme were given on alternate days as shown. From the 11th day on, each mouse was administered an amount of enzyme (symbols replaced by units given) calculated to bring its weight to, or maintain it at, its Day-0 value. Crosses = deaths of untreated controls (one was necropsied) and experimental animals. Each symbol = an individual mouse.

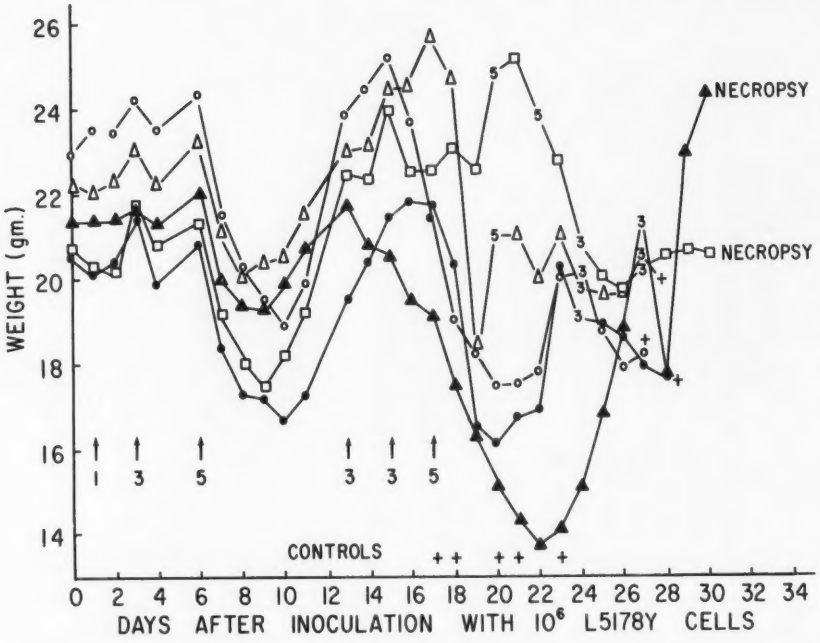

FIGURE 5.—Effect of PEG-L-glutaminase-L-asparaginase on the survival of BDF_1 mice infected ip with 10^6 L5178Y tumor cells. The enzyme was given ip at the times and dosage units indicated by the vertical arrows. Beginning on the 20th day, the mice were dosed individually on the basis of their body weights. Crosses = deaths of untreated controls and experimental animals. Each symbol = an individual mouse.

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In Vitro System for Detection of Antimetabolites of Specific Amino Acids [1]

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SUMMARY

An in vitro system was developed to detect antimetabolites in fermentation liquors of soil microorganisms. This system effectively uncovers antimetabolites of purines and pyrimidines and of selected amino acids with established differences in their biosynthesis by normal cells versus certain malignant cells. Currently these amino acids include: L-asparagine, L-aspartic acid, L-glutamine, L-cysteine (cystine), L-methionine, L-arginine, L-histidine, L-tyrosine, L-phenylalanine, L-tryptophan, L-threonine, and L-serine. It is expected that these antimetabolites could be useful either alone or in combination with specific depleting enzymes in the treatment of malignancies where such an imbalance was established.

[Cancer Treat Rep 63:1133–1136, 1979]

In the early 1960s, an in vitro screening system for the detection of antimetabolites was developed in our laboratories. This system was based on the observation that known antimetabolites inhibited the growth of a susceptible microorganism very strongly when it was cultivated in a minimal synthetic medium, while no inhibition (or at least much less) was observed on complex media, such as nutrient agar. In contrast, the effect of most of the antibacterial drugs is fairly comparable regardless of the cultivation medium used. The data presented in table 1 illustrate this point.

This observation was used in the development of a screening system for the detection of different antimetabolites in fermentation liquors of soil microorganisms such as *Streptomyces* or molds (1,2). The system was later utilized by a number of other laboratories in several countries (3,4). In this screening system, the fermentation liquors are tested in a disc-plate assay against a suitable microorganism, such as *Escherichia coli* or *Bacillus subtilis*, grown in a complex and also in a minimal synthetic medium. Samples that inhibit the sensitive microorganism more when it is grown in the synthetic medium are considered potential leads. In the next overnight experiment we try to determine whether the observed inhibition can be antagonized by incorporating several common metabolites into the minimal synthetic medium. The results of this study will give us early information about what type of antimetabolite is present in the sample tested (*see* table 2).

Obviously, the antimicrobial activity of sample 1 (table 2) was antagonized by adding a mixture of amino acids to the minimal synthetic medium. Sample 2 (table 2) was antagonized by some of the purines or pyrimidines present in the mixture. In the next two consecutive experiments, we first added groups of amino acids and then finally single amino acids to the minimal medium to find out which one specifically was antagonizing the antimicrobial activity. In our past experience, approximately 5% of the cultures tested had an in vitro spectrum typical of an antimetabolite, and, of these cultures, about 25% appeared to produce antimetabolites of some amino acid; this is a high percent, indeed (*see* table 3). It is important to realize that most cultures produce several in vitro active components, and that special techniques must be used early in our studies to make sure that only the desirable component will be isolated.

We should note that during our first period of screening for antimetabolites (1965–1970), any type of antimetabolite was followed and further tested. Later on, this system was further developed so that, since the summer of 1976, we have followed only cultures producing antimetabolites of purines and pyrimidines and of selected amino acids. Also, the in vivo activity is tested after partial

[1]Supported in part by contract N01-CM-77100 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

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**Reprint requests to:* Dr. Ladislav J. Haňka, Cancer Research Laboratories, The Upjohn Company, Kalamazoo, Mich 49001.

TABLE 1.—Inhibition by several drugs of 2 bacteria grown in 2 different media

Drug	Concentration (μg/ml)	Test organism	Zone of inhibition (mm) on— Nutrient agar	Zone of inhibition (mm) on— Minimal synthetic agar
Antimetabolites				
5-Fluoro-2'-deoxyuridine	5	*E. coli*	0	22
6-Azauracil	50	*E. coli*	0	24
5-Azacytidine	10	*E. coli*	0	25
Methotrexate	5	*B. subtilis*	0	40
AT-125	0.5	*B. subtilis*	0	50
Antibacterial antibiotics				
Dihydrostreptomycin	10	*E. coli*	18	17
Chloromycetin	5	*E. coli*	21	31
Neomycin	30	*E. coli*	19	22

TABLE 2.—Inhibition by 2 fermentation liquors of *E. coli* grown in different media

Sample No.	Zone of inhibition (mm) on— Nutrient agar	Zone of inhibition (mm) on— Minimal synthetic agar	Minimal synthetic agar further supplemented with— Combined amino acids	Minimal synthetic agar further supplemented with— Combined vitamins	Minimal synthetic agar further supplemented with— Combined purines and pyrimidines
1	0	27	0	24	26
2	0	29	27	30	0

purification of the fermentation liquors. These modifications resulted in a strong increase in the percent of in vivo active cultures as shown in table 4.

I should stress that at times during our past screening efforts we did not even look actively for antimetabolites of amino acids. There was not enough interest in these compounds, while antimetabolites of purines and pyrimidines were understandably the most desirable. Nevertheless, a number of amino acid antimetabolites were isolated over the years. Some were new drugs and some had been described previously. Two examples will be presented.

The first example is 2-amino-4-methyl-4-hexenoic acid (NSC-121238) (fig 1). This drug was a new antimetabolite of leucine; it had modest in vivo activity against the Walker 256 carcinosarcoma in rats (5). The activity of the second drug, AT-125 (NSC-163501) (fig 2), was antagonized by L-histidine in bacteria and by L-glutamine in cell culture. It is a novel drug, has demonstrated in vivo activity in several tumors, and is now ready for preclinical toxicology studies at the National Cancer Institute (6–8).

Two important considerations might influence our future work in this area. The first consideration is to determine what specific antimetabolites we should be looking for. The screen can be made very specific, as explained earlier. At present we are actively searching for the antimetabolites (analogs) of the following compounds: (*a*) all naturally occurring purines and pyrimidines and (*b*) select amino acids: L-asparagine (9–14); L-aspartic acid[3]; L-glutamine (15–18); L-cysteine (19,20); L-methionine (21); L-arginine (22,23); L-histidine (24); L-tyrosine (25,26); L-phenylalanine (27,28); L-tryptophan (29); L-threonine (30); and L-serine (31).

An amino acid should be put on this list if it meets any one of the following criteria:

a. a documented difference between malignant and normal cells with regard to biosynthesis of such an amino acid (on occasion, a substantially increased requirement rather than the lost capacity to synthesize such an amino acid might justify inclusion);

b. evidence that a depleting enzyme of such an amino acid has proven to be of therapeutic value in certain malignancies;

c. evidence that an analog(s) of such an amino acid demonstrates antitumor effects in vivo.

This whole area is changing rapidly, and new evidence

[3]Cooney DA. Personal communication, 1978.

TABLE 3.—Reversal pattern of 423 in vitro active cultures

No. of cultures in which antimicrobial activity was reversed (antagonized) by—			
Combined amino acids	Combined vitamins	Combined purines and pyrimidines	No. of cultures with no specific reversal
105 (25%)	69 (16%)	39 (9%)	210 (50%)

is being accumulated rapidly. Several of these amino acids have been added to our list within the last year.

The second consideration is a more complicated one. Antimetabolites can be applied in the chemotherapy for cancer either as single drugs or in combination. There are a number of antimetabolites being used clinically with success as single agents, particularly antimetabolites of purines and pyrimidines. While searching for these drugs, a requirement for early activity against an experimental animal tumor (P388) must be met. We intend to persist in this search and retain these criteria. However, we are now adding a fair degree of selectivity to our primary screen, for instance, by searching for antimetabolites of specific amino acids based on a biochemical rationale. Relatively little is known about the antitumor potential of these compounds apart from the antimetabolites of L-glutamine. Obviously, it would be very desirable to have some of the existing antimetabolites evaluated in vivo alone or in combination with the specific depleting enzyme and, hopefully, in other tumor systems besides leukemias. After more of such studies have been done, we would be in a better position to ask ourselves this question: is our current requirement that every potential lead be active in vivo against a murine leukemia justified, or should our increased degree of selectivity in the in vitro detection system (32) be paralleled by early use of new and perhaps more relevant experimental tumors? We realize that this is not an easy question to answer, but it ought to be considered seriously.

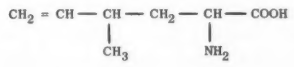

FIGURE 1.—Structure of 2-amino-4-methyl-4-hexenoic acid.

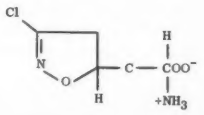

FIGURE 2.—Structure of AT-125.

TABLE 4.—Performance of the in vitro screen for antimetabolites during 2 time intervals

	Time intervals	
	1965–1970	1976–1977
Total No. of cultures tested	11,600	1537
No. of cultures—		
Active in vitro	585 (5%)	40 (3%)
Active in vivo	12 (2%)	9 (23%)

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Amino Acid Manipulations in Cancer Treatment: A Perspective From the National Cancer Institute Workshop

F. M. Muggia,* R. Catane, J. Douros, and D. Cooney[1]

Manipulations of specific amino acids have not been widely employed in cancer treatment. This does not indicate the neglect of possible nutritional factors modulating tumor growth, but rather the recognition of major methodologic problems which have arisen in trying to achieve or define the nature of amino acid depletion.

In this workshop, initially, the past and current clinical approaches to such manipulations were briefly reviewed. Subsequently, laboratory investigation into several areas involving amino acid depletion or the identification of amino acid analogs was presented, with indications of the many possible future avenues of therapeutic research. It was concluded that an integrated approach involving biochemists, tumor biologists, medicinal chemists, pharmacologists, and clinicians would probably be the most productive. Publication of this workshop should facilitate these aims and provide an overview of the therapeutic potential and possible problems related to amino acid manipulation in cancer treatment.

The ultimate aim of amino acid manipulations is to achieve a change in amino acid metabolism which will inhibit tumor growth with little or no deleterious effect on the host. Four possible mechanisms are available for achieving this objective.

The first possible mechanism is the administration of amino acid analogs which inhibit one or several specific enzyme pathways of amino acid metabolism. Among those covered in this workshop are: azaserine, DON, azotomycin, seleno-L-methionine, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, L-alanosine, and L-methionine-*dl*-sulfoximine. Information on other amino acid analogs with antitumor activity may be obtained from recent reviews (1–3).

The second is the administration of enzymes with amino acid degrading activity, which are therefore capable of rapidly depleting specific plasma amino acids, such as: L-asparaginase, L-glutaminase, L-asparaginase-L-glutaminase, L-tyrosine phenol lyase, L-phenylalanine ammonia lyase, L-threonine deaminase, L-cysteine desulfhydrase, and indolyl-3-alkane-hydroxylase. The third enzyme listed (L-asparaginase-L-glutaminase) is a mixed amidohydrolase capable of degrading both L-asparagine and L-glutamine at roughly equal rates. This enzyme (from *Acinetobacter*) has one unusual and advantageous feature: it does not provoke the emergence of resistant variants in susceptible animal tumors; by contrast, L-asparaginase from *Escherichia coli* does so rapidly. Inasmuch as all of the agents from this group are proteins, and hence potent antigens, a major issue discussed at this meeting was how to avoid immunogenic responses to those enzymes.

The third possible mechanism is the administration of other antineoplastic agents with indirect effects on amino acid metabolism. Two agents were referred to: 6-azauridine, discussed by Slavik (4), and *N*-phosphonacetyl-L-aspartic acid, discussed by Jayaram and Cooney (5). It is plausible that many anticancer agents, especially nucleic acid analogs, act by disturbing amino acid metabolism. Alterations in DNA and RNA may alter the structure of codons, resulting in the insertion of the wrong amino acid into proteins. Other changes may result from inhibition of de novo purine and pyrimidine metabolic pathways or from inhibition of the metabolism of co-factors which are important in amino acid metabolism.

And the fourth mechanism is the deprivation or imbalance of amino acids in the environment; dietary restriction may be an effective therapeutic modality in murine tumors (6). An excess of certain amino acids may also affect tumor metabolism.

Several areas of research were suggested at this workshop:

(*a*) The use of new animal models, eg, the use of human tumor xenografts implanted into nude mice as screens for therapeutic analogs or enzymes;

(*b*) the use of novel selection techniques for identification of new agents;

(*c*) chemical engineering to increase the biologic half-life and decrease the immunogenicity of specific depleting enzymes;

(*d*) further inquiry into the amino acid pathways of tumors and normal tissues with the stress on exploitable differences; and

(*e*) possibilities of the combined use of amino acid depletion and amino acid analogs.

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It is apparent that many methodologic problems of the past are yielding to advancing technology, and that studies in the above directions enhance the applicability of amino acid manipulations in cancer treatment.

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ANNOUNCEMENTS

Fourth Annual Symposium on "Rational Therapeutics"

Newport Beach, California, September 29–30, 1979

The Fourth Annual Symposium on "Rational Therapeutics" will be offered September 29–30, 1979, by the Department of Pharmacy Services, City of Hope National Medical Center. This advanced symposium has been designed to provide each participating member of the oncology team with the most current, clinically relevant information on various malignant diseases, their diagnosis, and available treatment modalities. This unique program will focus on: (1) soft tissue sarcomas, both childhood and adult; (2) endometrial carcinoma; (3) lung cancer; (4) current concepts of TPN as they relate to the cancer patient; and (5) cancer-related problems such as malignant effusions, infection, metabolic complications, pain relief, and the control of nausea. In addition, the symposium will have available six clinical tutorials covering problem areas in the overall management of the oncology patient. Each participant will have the opportunity to choose two of the six tutorials.

The faculty will include such speakers as, Donald Pinkel, MD, Cary Presant, MD, Samuel Ballon, MD, Michael Belman, MD, and William Hewitt, MD. Twelve hours of accredited continuing education have been applied for from the California State Board of Pharmacy and from the California State Board of Nursing. The fee for the 2-day seminar will be $90.00. The program site is the Newporter Inn, Newport Beach, Calif. ***Further information can be obtained by contacting:*** Thomas E. Saito, Program Chairman, Department of Pharmacy Services, City of Hope National Medical Center, 1500 E Duarte Rd, Duarte, Calif 91010.

American Board of Internal Medicine Examinations

The next examinations in Hematology, Infectious Disease, Nephrology, Pulmonary Disease, and Rheumatology will be on June 17, 1980. Registration for these subspecialty examinations will begin August 1, 1979, and will continue through November 1, 1979. For those who began their residency training in internal medicine before June 1, 1970, and who have < 2 years of acceptable subspecialty training this will be the last opportunity for admission to a subspecialty examination. ***Applications may be obtained from:*** The American Board of Internal Medicine, University City Science Center, 3624 Market St, Philadelphia, Pa 19104 (telephone: 215-243-1500).

The American Board of Internal Medicine has announced a significant change in policy regarding its training requirements. This change will affect physicians desiring admission to a subspecialty examination who began their residency training in ***internal medicine*** before June 1, 1970. At present, these physicians are considered to have met the training requirements for admission to a subspecialty examination after completing 1 year of acceptable training in the subspecialty. If once informed of admission to a subspecialty examination, these physicians ***will continue*** to be considered to have fulfilled the Board's training requirements in that subspecialty. Beginning with the subspecialty examination offered in 1981, however, ***all physicians*** not previously admitted to an examination will be required to meet the same training requirements which apply to physicians beginning their residency training after June 1, 1970. These requirements currently involve 2 full years of acceptable training in the subspecialty. A complete description of the Board's policies and procedures may be obtained by directing a written request to the American Board of Internal Medicine (above address).

American Board of Internal Medicine Re-Certification Examination

The third re-certification examination in internal medicine of the American Board of Internal Medicine will be administered at test centers throughout the US on Saturday, October 25, 1980. Any certified internist who was certified or re-certified in internal medicine prior to 1975 is eligible to take this examination. The purpose of the re-certification examination in internal medicine is to provide the practicing certified internist an opportunity

to demonstrate his or her current clinical knowledge and problem-solving abilities which relate to the provision of excellent care in internal medicine. The Board administered re-certification examinations in 1974 and 1977, and 5400 internists have already been re-certified.

The re-certification examination will be a comprehensive, full-day, proctored examination. It will consist of a one-half-day session of multiple-choice questions and one-half day of sequential latent-image patient management problems. All of the questions will relate to clinical information in general internal medicine with an emphasis on recent advances in internal medicine. Much of the examination will reflect the content of the syllabus and the questions in the American College of Physician's Medical Knowledge Self-Assessment Program (MKSAP V).

The fee for the examination is $175; applications for the examination will be accepted *November 1, 1979, through June 1, 1980.* For application forms write to the American Board of Internal Medicine, 3624 Market St, Philadelphia, Pa 19104.

Program on "The Current Issues in Pediatric Oncology Nursing"

Chicago, Illinois, September 20–22, 1979

The Association of Pediatric Oncology Nurses will be sponsoring a 3-day program entitled "The Current Issues in Pediatric Oncology Nursing" on September 20–22, 1979, in Chicago, Illinois. The fees are $45 for students, $60 for Association of Pediatric Oncology Nurses members, and $75 for nonmembers. *Interested persons should contact:* Margaret Stewart, RN, Department of Hematology, Children's Memorial Hospital, 2300 Children's Plaza, Chicago, Ill 60614.

Subspecialty Training in Hematology/Medical Oncology

The Army Medical Department provides subspecialty training in Hematology/Medical Oncology at five of its major medical centers (Brooke Army Medical Center, San Antonio, Texas; Fitzsimons Army Medical Center, San Francisco, California; Madigan Army Medical Center, Tacoma, Washington; and Walter Reed Army Medical Center, Washington, DC). Nine positions are available for 1st-year positions. Applicants must be US citizens and must be board-eligible/certified in Internal Medicine. Excellent salary and fringe benefits are available. *For further information contact:* Daniel B. Kimball, Jr, MD, Room 2J05, Building 2, Walter Reed Army Medical Center, Washington, DC 20012, or call collect (202-576-1208). The application deadline is *October 1, 1979.*

Symposium on "The Diagnosis and Treatment of Bone Tumors"

New York, New York, November 17–18, 1979

The Memorial Sloan-Kettering Cancer Center is organizing a symposium on "The Diagnosis and Treatment of Bone Tumors" to be held at the Memorial Hospital, New York, NY, on November 17–18, 1979.

Experts in the various disciplines will present the latest available information on the pathology, radiographic diagnosis, scintigraphic imaging, and management of the patient with benign and malignant bone tumors at various stages of the disease. The importance of the modern interdisciplinary approach in the treatment of bone tumors will also be explored.

Attendance is open to physicians only. There will be a $200 registration fee with advanced registration; this is mandatory due to limited space availability. This continuing medical education activity meets the criteria for 13 credit hours in Category I of the Physician's Recognition Award of the American Medical Association. *For further information write:* A. G. Huvos, MD, Symposium Coordinator, Memorial Hospital, 1275 York Ave, New York, NY 10021.

Midwest Cancer Seminar

Madison, Wisconsin, September 20–22, 1979

The Midwest Cancer Seminar is being held in Madison, Wisconsin, September 20–22, 1979, at the Concourse. It has been planned to bring to the practicing physician current knowledge regarding screening, diagnosis, and management of major malignancies and to emphasize the practical application of new methodologies. An outstanding faculty will be presenting the program.

This seminar is sponsored by the Wisconsin Clinical Cancer Center of the University of Wisconsin, Center for Health Sciences, Madison; Midwest Children's Cancer Center of the Medical College of Wisconsin, Milwaukee; American Cancer Society-Wisconsin Division, Inc, Madison; Eastern Cooperative Oncology Group; and Cancer Education Committee of Wisconsin Clinical Cancer Center. As an organization accredited for Continuing Medical Education, the Wisconsin Clinical Cancer Center certifies that this continuing medical education activity meets the criteria for 12 credit hours in Category I of the Physician's Recognition Award of the American Medical Association. This program is also acceptable for 12 prescribed hours by the American Academy of Family Physicians.

In addition, tickets will be available for the Wisconsin-UCLA football game. *For further information and a registration form please contact:* Paul C. Tracy, MD, Wisconsin Clinical Cancer Center, 1900 University Ave, Madison, Wisc 53705.

Monograph on Non-Hodgkin's Lymphoma

A limited number of copies of the collected papers from the National Cancer Institute-CCIRC symposium on non-Hodgkin's lymphomas which was held in San Francisco, Calif, in 1976, and published in *Cancer Treatment Reports* (vol 61, No. 6), are still available without charge. *Please contact* Stephen E. Jones, MD, Section of Hematology-Medical Oncology, University of Arizona Cancer Center, Arizona Health Sciences Center, Tucson, Ariz 85724, in writing for a copy.

CORRECTIONS

The authors of the article "Combined DTIC and *cis*-Dichlorodiammineplatinum(II) Therapy for Patients With Disseminated Melanoma: A Northern California Oncology Group Study" which appeared in the March 1979 issue of *Cancer Treatment Reports* regret that Dr. Harry Drasin's name was inadvertently omitted from the list of authors for this paper.

The editorial staff of *Cancer Treatment Reports* regrets that an error appears in figure 3 of the article "Pharmacokinetics and Metabolism of Ftorafur in Man" by Au et al which was published in the March issue (Vol. 63, No. 3). At the top of the figure, the explanation of the symbols is incorrect and should have been deleted. We apologize to the authors and the readers for this error.

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